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Photometric Estimation of the Relative Amount of DNA in Early Chick Embryos

By

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Abstract

EMANUELSSON, H. *Photometric estimation of the relative amount of DNA in early chick embryos.* Acta physiol. scand. 1961. *52.* 197—210. — Using the two-wavelength method the author has determined photometrically the amount of DNA in interphase nuclei of cells from chick blastoderms, 0—40 hours of incubation. The investigation was performed on sectioned material, stained according to the Feulgen method. Comparison of results for the different developmental stages shows that essentially only minor variations exist between the stages. Exceptions are the yolk endoderm cells at the 40-hour stage which apparently are polyploid and cells from 5-hour-blastoderms which have an average of 40 per cent more DNA, than that found in other stages. In combination with a previous observation of a high level of DNA in early chick blastoderms the present result indicates a temporary occurrence of cytoplasmatic DNA in the chick embryo. The high DNA-value at the 5-hour-stage is interpreted as indicating a partial synchronism in cell-division during the early development of the embryo.

In a previous investigation, EMANUELSSON (1958), the author has reported indications of a remarkably high amount of DNA in the cells of the early chick embryo. Thus for the first 18 hours of development it seems that the chick blastoderm has an average amount of DNA per cell in the embryo area which is double the amount recorded for cells from the same area later in development. As the analyses gave no information concerning the localization of this exceeding amount of DNA, it remained to be settled whether the high level of DNA reflects a widespread polyploidy in the early embryo cells or whether it possibly can be ascribed to a temporary occurrence of cytoplasmatic DNA.

As for the latter so many indications of the occurrence of cytoplasmatic DNA have been reported in the literature up to now that its existence scarcely can be

questioned any longer. Little is known about the chemical nature of it; the cytoplasmatic DNA reported for the amphibian egg is in fact thought to be desoxyribosidic compounds, acting as DNA-precursors, HOFF-JØRGENSEN and ZEUTHEN (1952), GREGG and LØVTRUP (1955), BIEBER et al. (1959).

Polyploidal cells, on the other hand, are not uncommon in animal embryos, as has been revealed by photometric investigations on isolated nuclei. Special attention may be called here to MOORE's (1957) investigation on frog embryos in which she demonstrates a striking polyploidy in her material, there being represented nuclei with up to the octoploid amount of DNA.

In early chick embryos, incubated under normal conditions, the present author has occasionally observed tripolar spindles in the dividing cells, but obviously there is a very low frequency of these. Otherwise there have been no cytological indications of polyploidy in these embryos.

To elucidate the question and settle whether an elevated DNA-level in the earliest stages can be attributed to an especially high amount of nuclear DNA or not, it was finally decided to compare chick embryos at different stages of the early development with reference to the amount of nuclear DNA in the cells. For that reason photometric determinations of nuclear DNA have been performed on cells from early blastoderms, which after sectioning were stained according to the FEULGEN method.

Material and Methods

Preparation of the material

The experimental material employed was fertilized eggs of pure breed (White Leghorn), laid the day before.

In some of the eggs the blastoderm was removed prior to incubation and immediately fixed (0-hour-stage). Other eggs were incubated at 37.5° ± 0.5° C for various lengths of time before removal and fixation of the blastoderms (5-, 14—16-, 18-, and 40-hour-stages). Fixative: absolute alcohol + glacial acetic acid (3 : 1), held at + 4° C. The classification of the developmental stages is made in accordance with the system of Hamburger and Hamilton (1951). Control embryos, their incubation extended to 14 days, showed a quite normal development.

The blastoderms were left in the fixative for 20 hours, then after dehydration and passage through methyl benzoate and benzene they were embedded in paraffin. They were serially sectioned at 8 and 12 μ respectively. The Feulgen staining was made according to LEUCHTENBERGER (1958), the time of hydrolysis being set at 12 minutes. All slides were stained in direct succession, and care was taken to carry out an absolute uniform treatment of the slides. The mounting medium was DePeX (Gurr). The cover glasses as well as the slides used were of uniform thickness.

Many investigators have successfully used the Feulgen reaction for quantitative determinations, cf. LEUCHTENBERGER (1958), SWIFT (1953), LESSLER (1953), KURNICK (1955). But obviously the intensity of the stain can vary with the fixative, with the time of hydrolysis and with different methods of preparation of the stain. However, it may be assumed that in the present investigation the preparations are not subject to such errors, but are all mutually directly comparable, as all blastoderms have been prepared identically and in direct succession.

Photometric Feulgen determination

Evaluation of relative amount of DNA (chromophore) in the nuclei is based on the two-wavelength method. For theoretical discussions of the latter see, e. g., ORNSTEIN (1952) and PATAU (1952). The advantage of the two-wavelength method compared with the single-wavelength methods is that the former is much less sensitive to a heterogenous distribution of the chromophore to be measured; further, it does not require the object in sharp focus. The two wavelengths chosen in the present investigation, when dealing with Feulgen-stained material have been 519 mμ and 570 mμ respectively. Thus the photometric measurements on the pure Feulgen stain and on stained nuclei showed that these two wavelengths fulfil the requirements of the method, i. e., extinction at one of them is twice the extinction at the other.

The photometric measurements on the investigated embryo-material have been carried out with the aid of a microspectrophotometer (the details of which are given below).

Estimation of the total amount of chromophore in a single nucleus involves securing the transmission values at the two wavelengths stated for a circular field including the selected nucleus. The diameter of this field, which may be regulated by means of the ocular-diaphragm, was held constant in all the measurements.

From the transmission values the relative amount of chromophore is then calculated according to PATAU's formulae.

Thanks to MENDELSOHN (1958), who has published a very useful set of tables based on PATAU's formulae, actual calculation is made unnecessary as the amount of chromophore can be derived in the tables directly from the transmission values. According to the same author only those values are included in the tables which fall within the effective range of the two-wavelength method. The transmission values found in the present investigation have all been within the scope of the tables in question.

The photometric measurements extended over many days. Therefore, as a precaution, samples of nuclei representing all the different stages were finally measured in rapid succession over short intervals. The latter values were in strict conformity with earlier results.

Micro-spectrophotometer

The micro-spectrophotometer used is of conventional design and based on the same principles as described by, e. g., LEUCHTENBERGER (1958), SWIFT and RASCH (1956) and MENDELSOHN (1958).

Light source. A Philips tungsten ribbon-filament lamp (10 V, 16—17 Amp) operated at 15 Amp was the light source used. The light goes via a flint-prism monochromator (Carl Zeiss, Berlin-Steglitz), the exit slit of which is replaced by a Pulfrich diaphragm (Zeiss, Jena) projecting a square lightspot via a mirror and an achromatic sub-stage condensor in the image plane.

Optics. The microscope was a Zeiss-Winkel standard microscope equipped with an achromatic sub-stage condensor (COOKE, TROUGHTON and SIMMS n. a. 1.0) operated at low aperture (c. 0.3). For orientation in the slide the monochromatic light can be replaced by a built-in light-source. Objective used: a C. Zeiss immersion-objective (Zeiss Apo 100/1.32 Öl). Immersion oil: refractive index 1.5150. A Ramsden-ocular with built-in iris-diaphragm makes it possible to limit the area projected on the photo-cell.

Light-registration. Over the microscope a micro-photography camera bellows is adjusted in a horizontal swinging position and at registration the light is projected on a photo-cell (RCA IP21) placed on top of the bellows. For centering the light spot on the photo-cell, the photo-cell-housing can be replaced by a ground-glass plate. The photo-cell is fed by a high-voltage stabilisator, in our case operated at 960 V, and readings are made on a Multiflex spot-galvanometer.

Main supply. All electric equipment is connected to the main supply via a Philips-stabilisator (500 VA) giving an output voltage of 220 ± 1 V at 195—255 volts input.

Actual tests with the photometric equipment have shown that the Schwarzschild-Villiger effect, NAORA (1952), is negligible in the present investigation.

It is difficult to estimate the errors associated with the two-wavelength method, as yet comparatively few investigators have applied this method for DNA-measurements and consequently sufficient material for a direct comparison is not available. Measurements undertaken by MENDELSOHN (1958) and MENDELSOHN and RICHARDS (1958) seem to indicate, however, that errors inherent in the two-wavelength method are indeed very small, and it may be assumed that they fall below the error margin of ordinary photometric DNA-measurements at a single wavelength. To the latter type of measurement a maximal error of 15 per cent has been ascribed, SWIFT (1953), LEUCHTENBERGER (1954).

At no stage was staining of the cytoplasm observed. The measurements have shown that not even at the earliest stages (0-hour and 5-hour blastoderms) when yolk granulae in the cells are very apparent, is there any measurable variation in amount of chromophore which may possibly be ascribed to the presence of these granulae.

In a sectioned material there is risk of including slightly injured or overlapped nuclei among those selected for measurement. However, provided the diameter of the cell nuclei is of approximately the same size for all the investigated stages — and on the whole that seems to be the case for the cells of the embryo area — this error will be of fairly constant size for sections of the same thickness. The mean DNA-values of blastoderms sectioned at 8 μ have constantly been c. 5 per cent lower than in blastoderms sectioned at 12 μ. The difference reflects the higher percentage of injured nuclei occurring in the thinner sections, cf. IVERSEN (1960).

Results

All measurements are made on interphase nuclei. For each of the investigated embryo stages the individual DNA-values (chromophore values) have been arranged in histograms which are represented in Fig. 1—3. The mean DNA-value is given as well. The histograms refer to blastoderms sectioned at 12 μ. Each histogram is made up from values obtained from 2—3 blastoderms which among themselves showed closely agreeing distribution. As it is seen the individual nuclear values differ considerably, but even if a minor part of the variation must be assigned to errors inherent in the applied methods, it may be confidently assumed that the found differences essentially reflect a real variation in DNA-content of the nuclei.

0-hour-stage

At this stage, which forms a resting period before the onset of incubation, activity in the blastoderm is low and mitotic figures are sparse. Even if the found DNA-values are considerably scattered, there is in the histogram (Fig. 1) an accumulation of values around the chromophore value 180 as well as another slightly smaller accumulation around the value 300. Of these the former may be considered as representing the mean DNA-value of the resting interphase nucleus, i. e., the mean diploid value.

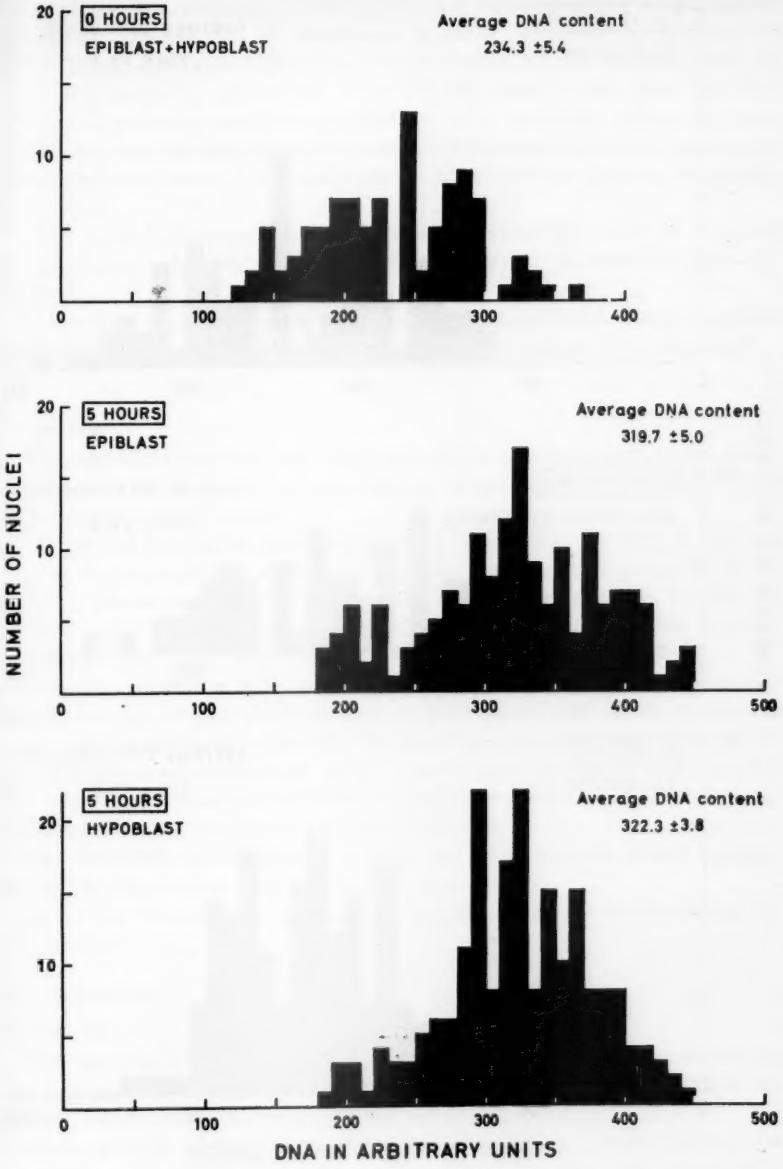

Fig. 1. Distribution of DNA-values in chick blastoderms sectioned at 12 μ.

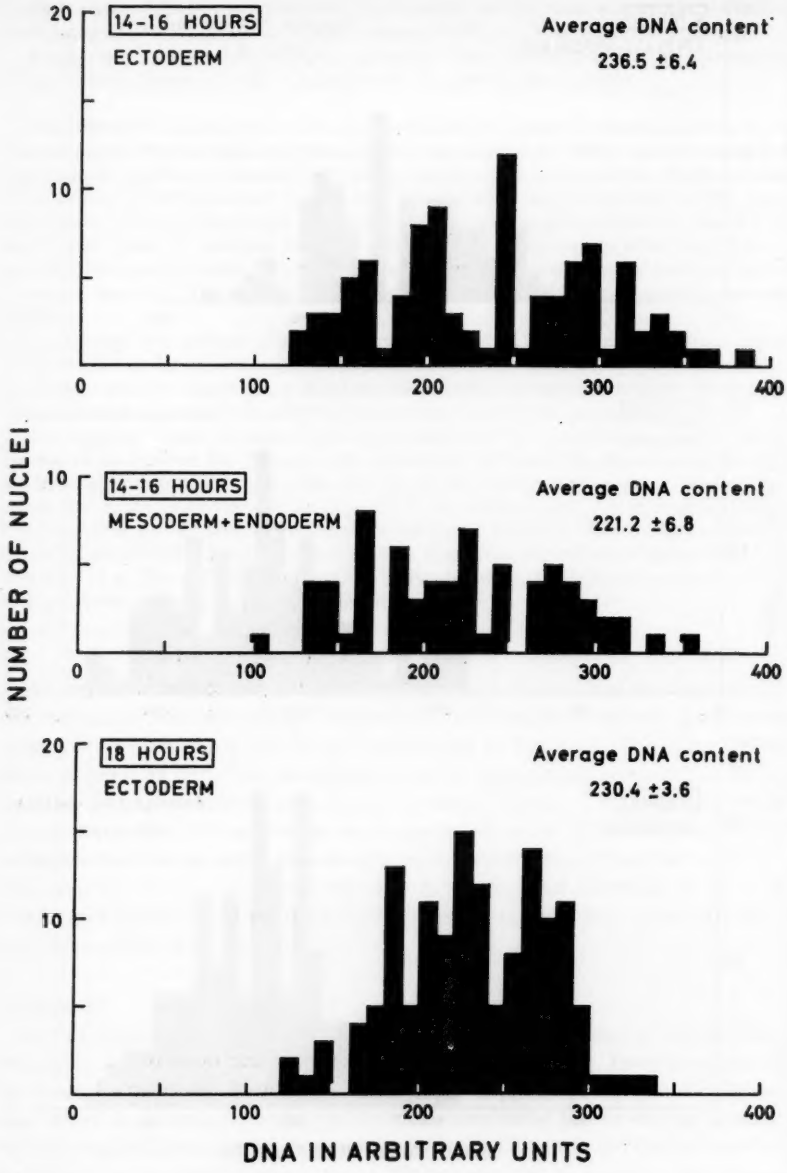

Fig. 2. Distribution of DNA-values in chick blastoderms sectioned at 12 μ.

As it has been shown by SWIFT (1953) and others doubling of the amount of nuclear DNA occurs in interphase. It seems reasonable to suppose that in the histograms values higher than 180, but below 360, are obtained from cells gradually preparing for mitosis. Now the physiological zero point for chick embryos is generally considered to lie at 20—21° C, EDWARDS (1902). However, metabolic processes undoubtedly occur in chick blastoderms also at temperatures below this point, even if the actual process of cell-division seems to be strongly suppressed.

The sparse occurrence in the histograms of values two times the supposed diploid mean value fits well with the low frequency of mitotic figures. Cf. Table I.

There exist no differences in the amount of nuclear DNA between epiblast (ectoderm cells) and underlying cells (hypoblast or endoderm) at this stage.

5-hour-stage

The picture given by the two histograms (Fig. 1) is at first somewhat puzzling, as the mean DNA-value is of quite another magnitude than that met with in other developmental stages.

To account for this we must first consider that during the stage in question a general reorganization to a new metabolic equilibrium is going on in the embryo. Mitotic activity which until recently was suppressed by the low environmental temperature has started again to a full extent, and there is reason to believe that the majority of cells are preparing to enter cell-division.

The same conclusion will be arrived at from the histograms if we persist in considering the mean diploid value as ranging around 180. In the histograms major accumulations of values are thus found around the chromophore values 300 and 400 respectively, and only a minor concentration is found around 200. The higher values may be taken as representing widespread DNA-duplication immediately preceding the actual cell-division.

The possibilities for the embryo to bring out such a general, almost synchronous DNA-duplication will be discussed later.

As for the 0-hour-stage both germ layers show the same distribution of the DNA-values.

14—16-hour-stage

18-hour-stage

The histograms (Fig. 2 and 3) are essentially of the same shape as for the 0-hour-stage, but a more pronounced concentration of values around the 180 value is found for the underlying layers.

Nuclei with the suggested tetraploid amount of DNA are almost lacking, but a significant number of the nuclei are just increasing their amount of DNA before mitosis.

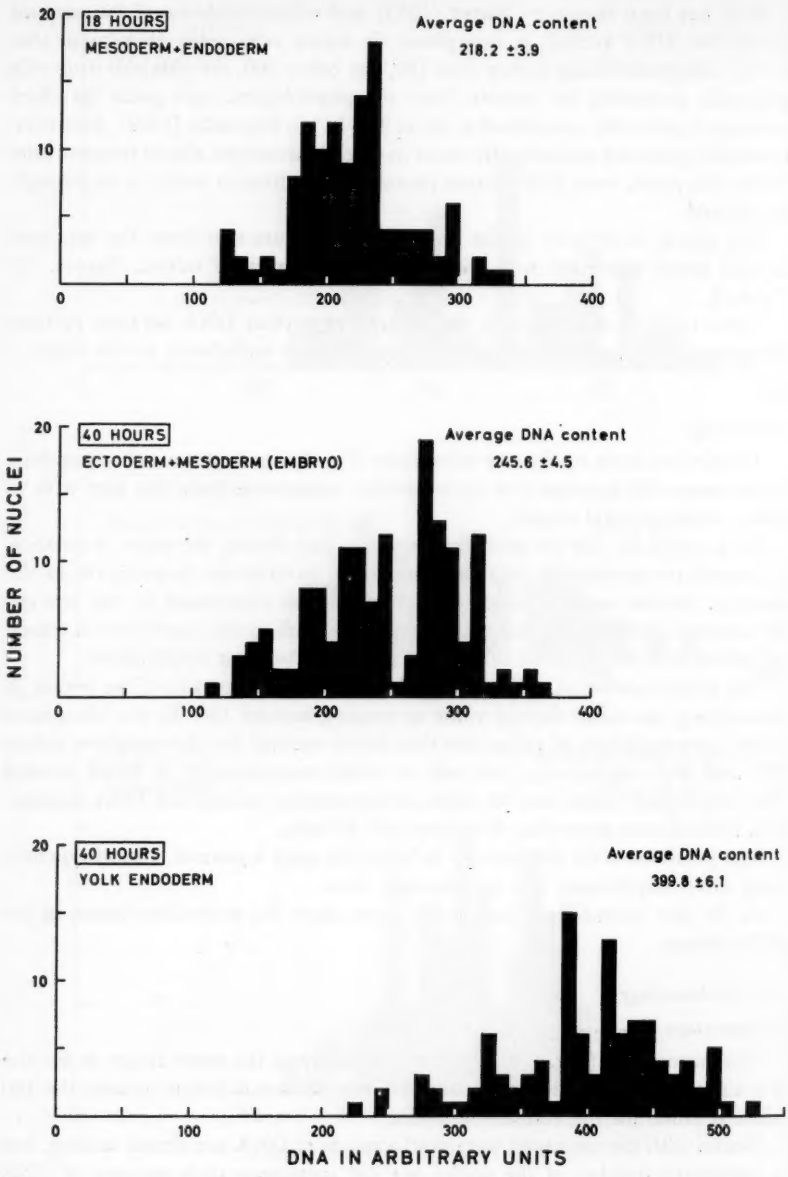

Fig. 3. Distribution of DNA-values in chick blastoderms sectioned at 12 μ.

Table I. Mitotic activity in early chick embryos

Mitotic counts were made in the same sections, 12 μ thick, that first had been submitted to photometric estimation of nuclear DNA. To avoid double counting alternate sections were omitted in the determination. The standard error of the mean has been calculated according to the formula $\pm \sqrt{\frac{p(100-p)}{N}}$, where p = mitotic index, N = number of investigated cells. Reported indices are in all cases derived from 2 separate blastoderms, which among themselves showed closely agreeing values.

Age of the embryo	Tissue	Mitotic index (per cent)
0 Hours	Epiblast + Hypoblast	0.79 ± 0.14
5 Hours	Epiblast	3.62 ± 0.30
	Hypoblast	3.80 ± 0.36
18 Hours	Ectoderm	3.72 ± 0.26
	Mesoderm + Endoderm	2.27 ± 0.22
40 Hours	Neural tube	4.70 ± 0.60
	Yolk endoderm	1.49 ± 0.26

40-hour-stage

At this advanced stage of development (Fig. 3) the embryo area still shows much the same pattern as was found in 0-hour and 14—18-hour blastoderms, but cells from the extraembryonic portion of the blastoderm present an entirely diverse pattern with a mean DNA-value near 400. Further inspection of the extraembryonic cells proved that these higher DNA-values are restricted to yolk endoderm cells, whereas the pattern for the ecto- and mesoderm cells is essentially the same in the extraembryonic as in the embryo area.

Except for this last mentioned stage (40 hours) distribution of DNA values within a germ layer is apparently much similar for the embryonic and extraembryonic portion of it.

The main result obtained is that during the investigated period of the development the chick embryo cells hold practically the same average DNA-content in the nuclei. The exceptionally high DNA-level at the 5-hour-stage is possibly of short duration.

To find out how close a connection there may be between distribution of nuclear DNA and apparent mitotic activity in the analyzed sections, mitotic indices have been calculated for some of the blastoderms. These values are given in Table I. It appears that mitotic indices are not differing when the germ layers show the same distribution of DNA-values (5-hour-stage). If the distribution is apparently dissimilar, however, the indices deviate too (18-hour-

stage). The higher index is then found in the layer having the higher portion of nuclei in the midst of DNA-synthesis. In the 18-hour-blastoderm the differences between the germ layers in mitotic index and DNA-distribution possibly suggest a prolongation of interphase in meso- and endoderm cells as compared with the ectoderm cells.

Obviously the comparatively low mitotic indices (3.6—3.8 per cent) for the 5-hour-stage are difficult to account for in the present case, as they were expected to be definitely higher than any value for the 18-hour-stage. Preliminary investigations indicate, however, that slightly older embryos (estimated at 5—8 hours) have a somewhat higher average value for the epiblast (about 5 per cent). Presumably this small but apparently significant increase bears direct relation to the deviating DNA-distribution met with in the 5-hour-blastoderm.

Discussion

The theory of DNA-constancy, according to which the amount of DNA per chromosome set is constant for an organism, has won widespread acceptance and SWIFT (1953) in his review on the subject concludes that the theory has been adequately confirmed. He admits, however, that the constancy in question may break down during the differentiation of cells. Discussing her own results from DNA-measurements on nuclei from frog embryo hybrids, MOORE (1957) stresses the fact that the DNA-constancy has not been established for embryonic cells and quotes results from vertebrate as well as invertebrate embryos.

In the present investigation the histograms are of an essentially similar shape, consistent with the constancy theory. However, the low frequency of high (tetraploid) DNA-values — especially for the 14—18-hour-stage — is confusing. Otherwise the shape of the histograms — with a main peak at the diploid value and a lower subsidiary peak at twice this value — is apparently what is to be expected in rapidly proliferating tissues, BLOCH (1958).

Two stages, the 5-hour-stage and the 40-hour stage show some peculiar features, which require further comment.

At the 40-hour-stage the yolk endoderm cells with their mostly tetraploid amount of DNA in the nucleus stand out distinctly against the cells of the embryo body. Along with the tetraploid value higher and also lower values are represented, but the shifting towards higher values is predominant and, strikingly enough, nuclei with the diploid amount of DNA are missing.

Closer inspection of the yolk endoderm cells in mitosis revealed that all the mitotic figures observed differ in some way from the normal type, often displaying apparently defect chromosomes. Mitotic disturbances in the yolk endoderm cells have earlier been reported by JACOBSON (1938), and also in his opinion the abnormal mitoses are a characteristic feature of the cell type in question. Judging from the investigated slides, the whole layer of yolk endoderm cells will most likely degenerate ultimately, a phenomenon which may

bear some relation to the simultaneous outgrowth of the vascular system. As to the nature of the mechanism that prevents the endoderm cells from cell-division without blocking their DNA-synthesis, nothing definite seems to be known. However, cell degeneration, often on a large scale, is apparently a normal phenomenon in vertebrate embryos, GLÜCKSMANN (1951). Substances released in cell degeneration in embryos probably exert an inducing effect, STOCKENBERG (1937).

Interestingly enough a cytological picture, very similar to that in the yolk endoderm at the 40-hour-stage, was observed by the author in the extraembryonal portion of 18-hour-blastoderms upon treatment with ribonuclease (RNA-se solution injected in the egg directly under the blastoderm).

The remarkable distribution of DNA-values at the 5-hour-stage with most of values located above the diploid level has been interpreted here as indicating that DNA-duplication is nearly synchronous in the majority of the blastoderm cells. The possibility of there existing instead just at the 5-hour-stage a population of nuclei with 300 as the mean diploid value seems less plausible. The distributional patterns of DNA-values from preceding and succeeding developmental stages (0-hour- and 14—16-hour-stage) lend no support for such an assumption.

However, a synchronous DNA-duplication on a large scale, as just outlined, can scarcely be accomplished by the blastoderm cells at this early stage without access to either stored DNA or stored desoxy-compounds, directly utilizable in the DNA-synthesis. The latter would rather be of desoxyribonucleoside-type, as according to KORNBERG (1959) the immediate precursors for DNA-synthesis are desoxyribonucleoside-triphosphates.

In the investigation that was referred to in the introduction, EMANUELSSON (1958), the author has shown that calculation of the average amount of DNA per cell for the embryo area in early chick blastoderms gives values for the 0-hour-stage and the 18-hour-stage which far exceed the value for the 26-hour-stage. The latter value, as well as values for more advanced stages are, however, in complete accordance with average values for chick embryos, reported by other investigators, e. g., DAVIDSON and LESLIE (1950).

The higher values alluded to above were obtained from biochemical analyses in combination with meticulous counting of cells of the corresponding area, but they were unfortunately rather incomplete. However, additional observations have given similar results, Fig. 4, and therefore the found deviation has been considered reliable.

As the present investigation has shown that the average amount of DNA per nucleus is practically the same for the 0-, 18- and 40-hour-stages, it is concluded that an elevated amount of DNA at the earliest stages probably is due to a stored, extra-nuclear supply of DNA. Even if this supply is rather small at the 0-hour-stage, it seems just big enough to permit such an almost simultaneous cell-duplication at about the 5-hour-stage as is now suggested. A temporarily

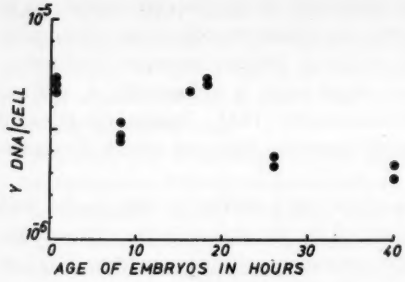

Fig. 4. Average amount of DNA per cell in the embryo area of early chick blastoderms The values are derived from biochemical analyses and cell counts for the area in question For further details *cf.* EMANUELSSON (1958).

raised demand for DNA, which was to be foreseen then, is also indicated in figure 6 by the decrease of the DNA level at about the actual stage.

As is seen in Fig. 4 there follows upon the decrease a replenishment of DNA to the original level, presumably effected by stored DNA-precursors, but from the 26-hour-stage on the exceeding amount of DNA is apparently exhausted and the embryo has switched over entirely to DNA-synthesis de novo. It seems likely that the last DNA-reserve is being used during that period of increased mitotic activity which in fact occurs in the embryo area at about the 20-hour-stage.

From HOFF-JØRGENSEN's (1954) investigations it is known that the whole hen's egg is rich in DNA. Furthermore the proposed scheme of an altered DNA-metabolism in the early chick embryo has its direct parallels. It is known that a shifting during embryogenesis from consumption of stored DNA or immediate DNA-precursors to DNA-synthesis de novo occurs in frog embryos, HOFF-JØRGENSEN and ZEUTHEN (1952), GRANT (1960) and in sea urchin embryos, AGRELL and PERSSON (1956). In the latter case it was indeed demonstrated that the embryo cells divided synchronously up to that developmental stage when the DNA-supply was exhausted and synthesis de novo started.

The figures in table I show that the mitotic index gives little support to the proposed scheme of a partial accumulation of cell division to the 5-hour-stage. However, it is difficult to predict how much the absolute value of the mitotic index may be influenced from the shifting DNA-distribution, as we do not know the length of the period during which interphase nuclei have a DNA-content, twice the diploid amount. It seems likely that the mitotic index is not much affected, if the last mentioned period is appreciably longer than the time for mitosis — a condition which possibly prevails at the 5-hour-stage.

Circumstantial evidence in favour of a partial synchronism in cell division in early chick embryo cells has also been reported earlier, EMANUELSSON (1958), and include principally a) rhythmical variation in the average DNA- and RNA-concentrations of early chick embryo cells, b) a varying DNA-synthesizing ability of early embryos, explanted at different stages.

The possible existence of a periodicity of cell division in chick embryos has been discussed by earlier investigators, of whom STOUGH (1935) may be especially mentioned. In their opinion there is no evidence of any periodicity. However, this view of the question is easily explained by the fact that the investigated embryos almost invariably have been older than 20 hours. Thus a highly important period actually has been missed.

DERRICK (1937) in her investigation of the mitotic index in the early chick embryos has drawn no conclusions regarding periodical variations in the average mitotic index, but valuable information bearing just upon this problem can be obtained from her values. They reveal that for all germ layers the average mitotic index is noticeably high in embryos at about the 4-hour-, the 20-hour- and the 5—8-somite-stage respectively. This finding is apparently in fairly good agreement with a suggested periodicity of cell division in the chick embryo.

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Mitotic Activity in Chick Embryos at the Primitive Streak Stage

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Abstract

EMANUELSSON, H. *Mitotic activity in chick embryos at the primitive streak stage.* Acta physiol. scand. 1961. *52.* 211—233. — In the present work it has been investigated whether previously recorded regional differences in the average RNA-concentration of the primitive streak embryo are also connected with differences in mitotic activity. Mitotic indices calculated for the embryo area reveal no decided regional dissimilarities. However, owing to the uncertainty inherent in the interpretation of the index, it was decided to extend the mitotic counts to embryos in which a successive accumulation of mitoses had been produced by means of heat shock and the mitotic poisons, colchicine and oestradiol. From the obvious differences in mitotic activity which appear in such embryos it is clear that cell multiplication is greatest in a region of the embryo comprising the node and adjacent areas on both sides thereof. As differences in cell multiplication are moderate, however, it is presumed that the existing variation in RNA-concentration must be partly ascribed to differences in protein synthesis within the embryo, associated with the beginning differentiation of the embryo cells. Cytologic and morphologic changes caused by the heat shocks and the mitotic poisons are described.

Much work has been devoted to the study of morphogenesis in the early chick blastoderm, and the numerous papers dealing with this subject have recently been excellently reviewed by WADDINGTON (1952). Most investigators have studied the large-scale cell-movements, usually referred to as "streaming", which come into operation in the early blastoderm; but the mitotic activity, occurring at the same time, has also attracted considerable interest. However,

to what extent localized cell proliferation can be involved in the outgrowth of the embryo body does not seem very clear. Remarkably enough, the rapidly growing primitive streak, for instance, cannot possibly be considered as a center of cell-proliferation (PASTEELS 1943).

Describing variations in the concentration of nucleic acids in the early chick embryo, the present author (EMANUELSSON 1958), has demonstrated within the embryo area the existence of local variations in the quotient RNA/DNA (an expression for the relative concentration of RNA), which according to the author suggests variations in cell-multiplication. Thus low values of the quotient would denote lively cell-division while high values of the quotient would signify a lower mitotic activity, possibly with predomination of cell-differentiation within the region in question.

For chick embryos of 23—40 hours' incubation this relation between the RNA/DNA quotient and the mitotic activity apparently exists. This relation is substantiated by preliminary observations of the present author, from metabolic data (SPRATT 1952) and from mitotic indices, reported by, *e. g.*, DERRICK (1937) and BELLAIRS (1955, 1957).

Of great interest is the question of whether the relation holds also for the primitive streak embryo (18-hour-embryo), in which a visible differentiation has not yet occurred, even if the presumptive fate of the different regions already is well defined, cf. WADDINGTON (1952).

In fact, the mitotic indices reported by DERRICK (1937) for chick embryos of this age lend some support to the idea of a variation in mitotic activity, such as intimated from the nucleic acid quotients. However, DERRICK's indices for the primitive streak stage — up to now the only existing detailed information of the mitotic activity in the 18-hour chick embryo — can scarcely be adopted in the present case without some reservation. Even if they often indicate big differences between adjacent regions, it is difficult to judge when a difference is significant, as statistical analysis of the material is lacking. Moreover, these indices are calculated for each germ layer separately, whereas the RNA/DNA quotients referred to were obtained for pieces of blastoderm containing all the three germ layers.

It has therefore been of interest to make a further investigation of mitotic activities in the primitive streak embryo in order to obtain mean values for cell multiplication within small, restricted sections of the blastoderm. In the present investigation the position of these sections has been of primary interest, while their composition of cell-material from the different germ-layers has been of secondary importance.

As it is fully recognized that a local increase of the mitotic activity is not bound to result in an appreciably elevated mitotic index — even if that seems likely — attempts have also been made to obtain additional measures of the mitotic activity in the blastoderms, more elucidative than the ordinary mitotic index.

The present paper deals principally with experiments in which blockage of blastoderm cells, having entered mitosis, will lead to successive accumulation of mitotic figures and thus facilitate the discovery of any existing differences in cell-multiplication. In the experiments the mitosis process has been blocked by heat and by addition of mitotic poisons, colchicine and oestradiol.

Material and Methods

Incubation of the eggs (White Leghorn, pure breed) was carried out in a thermostatically controlled incubator at 37.5° C ± 0.5° C. The atmosphere of the incubator was kept suitably moist by placing dishes of water inside.

Normal Embryos

After incubation for the desired period the embryos were removed from the eggs and were immediately fixed in Carnoy's fluid (absolute alcohol + glacial acetic acid, 3 : 1). They were stained in Gomori's hematoxylin according to MELANDER and WINGSTRAND (1953) and were mounted in DePeX (Gurr). Some of the embryos were sectioned (10 μ), but usually they were mounted whole.

Heat-Shocked Embryos

Preliminary experiments had proved that the mass of a hen's egg is too big to permit rapid changes in temperature of the egg contents, a fact which precludes the possibility of short-time incubation of the eggs at widely differing temperatures. All experiments including variations of the incubation temperature of the embryos have therefore been performed exclusively upon embryos cultivated in vitro in Carrel-flasks of small volume. To ensure even temperatures incubation was arranged in a thermostatically controlled water-bath, the flasks being almost entirely submerged in the water. Changes of the water-temperature could be made rapidly; thus alternation between two widely differing temperatures, *e. g.*; 20° C and 44° C, was accomplished in about 3 min. The embryos were explanted to the Carrel-flasks at the primitive streak stage (18 hours of incubation according to the system of HAMBURGER and HAMILTON (1951)). The explants have constituted the anterior part of — alternatively the whole of — the embryo area, the latter being defined in an earlier investigation, EMANUELSSON (1958) (cf. also Fig. 1). They were cultivated on a basic medium of the following composition:

	per cent
NaCl	0.650
KCl	0.033
$CaCl_2 \cdot 6\ H_2O$	0.031
$Na_2HPO_4 \cdot 2\ H_2O$	0.007
KH_2PO_4	0.003
$NaHCO_3$	0.055
Glucose	0.850
Agar	0.420

Before addition of the agar, egg white was added: 3 parts of basic medium were mixed with 2 parts of a solution prepared by shaking one egg white vigorously with 50 ml medium. By addition of egg white to the medium it was possible to maintain satisfactory mitotic activity for up to 48 hours in the explants. The explants were fixed in Carnoy's fluid or methanol; as stains were used Gomori's hematoxylin, gallocyanin-chromalum and Giemsa's solution. Mounting medium: DePeX.

Treatment with Oestradiol and Colchicine

In the treatment *in vitro* explanted embryos were cultivated on the basic medium, described above (no egg white), to which was added oestradiol or colchicine. As before the embryos were explanted at the primitive streak stage, the explant constituting the whole embryo area. Incubation was carried out at 37.5° C ± 0.5° C.

All experiments in vitro — also the heat-shock experiments — always have included controls, cultivated on the employed medium at 37.5° C.

For oestradiol a suitable concentration proved to be 11 μg/ml incubation medium. When adding the oestradiol to the basic medium it was necessary first to dissolve it in ethanol. Controls showed that the resulting very low concentration of ethanol of the medium (0.19 per cent) had no effect upon the cultivated embryos. With colchicine, which was dissolved directly in the medium, full effect was obtained in a concentration of 100 μg/ml cultivation medium.

Treatment with mitotic poisons *in ovo* was arranged so that after rubbing the egg with a pellet of cotton soaked in 70 per cent ethanol, a small opening was made in egg shell and shell membranes on the side of the egg, whereupon the drug, dissolved in 0.3 ml sterile 0.93 per cent NaCl-solution (37° C) was injected into the yolk, directly under the blastoderm. By using a curved hypodermic needle of small calibre it was possible to avoid piercing the vitelline membrane in the immediate vicinity of the blastoderm, and there was almost no escape of yolk out into the egg white. After the hole in the shell had been sealed with tape the egg was immediately restored to the incubator and placed with the "window" pointing sideways. Controls were injected with sterile NaCl-solution only.

Treatment in ovo was successful as far as it concerned colchicine, which was added in a dilution of $1 : 10^4$.

When using oestradiol in this type of experiment it was found that the concentration thereof had to be raised considerably over that used in the experiments in vitro. As it appeared that the concomitant increase in the ethanol concentration obviously had undesirable effects upon the embryo cells further experiments were suspended.

After treatment with mitotic poisons the embryos were fixed in Carnoy's solution, stained in Gomori's hematoxylin and mounted in DePeX. The embryo material comprised both sections (10—12 μ) and whole mounts.

Results

A. *Mitotic Indices in Normal Chick Blastoderms at the Primitive Streak Stage*

In Table I are given mitotic indices for normal chick embryos of 16—23 hours' incubation. As seen the figures refer to the whole embryo area as well as restricted parts thereof. For the primitive streak embryo (18-hour-embryo) the extent of these parts is outlined in Fig. 1.

The table permits comparison both between transversal and longitudinal strips of the embryo area. The transversal strips are numbered from the fore part of the embryo, so that part 1 includes the region before the streak, part 2 the anterior part of the streak with the node, and the remaining parts finally (3 and 4) comprise the central and terminal parts respectively of the streak. The 23-hour-embryo forms an exception to this: here the node is included in part 3. Three longitudinal strips are distinguished: one central and two lateral parts.

The mitotic index (the number of mitoses per 100 cells), reported for one of

Table I. Mitotic indices (per cent) for different parts of early chick embryos

The standard error of the mean is calculated according to the formula $\pm \sqrt{\frac{p(100-p)}{N}}$, where p = mitotic index, N = number of investigated cells

Part of embryo. Cf. fig. 1 A	Age of embryos					
	16 hrs	16—18 hrs	18 hrs	18 hrs	20 hrs	23 hrs
1	2.83 ± 0.30	3.75 ± 0.27	5.0 ± 0.45	5.20 ± 0.26	4.50 ± 0.29	3.06 ± 0.20
2	3.22 ± 0.25	4.06 ± 0.20	5.17 ± 0.28	6.40 ± 0.29	4.65 ± 0.31	4.30 ± 0.22
3	2.39 ± 0.27	3.86 ± 0.26	5.15 ± 0.29	5.17 ± 0.25	6.15 ± 0.24	4.47 ± 0.18
4	2.02 ± 0.33	3.75 ± 0.28	4.47 ± 0.26	4.60 ± 0.25	5.31 ± 0.24	4.39 ± 0.21
$L_1 + L_2$	2.70 ± 0.22	3.62 ± 0.17	4.70 ± 0.21	5.02 ± 0.23	5.03 ± 0.20	3.75 ± 0.15
C	3.25 ± 0.27	4.13 ± 0.17	4.72 ± 0.20	5.75 ± 0.20	5.57 ± 0.17	4.20 ± 0.14
1 + 2 + 3 + 4	2.89 ± 0.17	3.89 ± 0.12	4.70 ± 0.14	5.47 ± 0.15	5.35 ± 0.13	4.15 ± 0.10

these parts represents a mean value for the whole body of cells within it, irrespective of connexion to any specific germ layer. Each of the mean values is based upon examination of a great many cells, usually 3,000—6,000. By suitable staining (Gomori hematoxylin) cells and mitotic figures located at shifting levels within the preparation could be counted directly without sectioning of the material.

For the primitive streak embryo biochemical analyses had shown that those parts of the embryo area which here are denoted 1—4 have the following RNA/DNA-quotients: 7.3, 4.9, 7.1, 8.5. If the previously mentioned relation between this quotient and mitotic activity (see introductory part) is prevailing at this developmental stage too, the above values would imply that mitotic activity is high in that region of the embryo surrounding the anterior part of the streak, but lower in posterior regions as well as in the region in front of the streak. Similar variations in mitotic activity are also expected in immediately preceding and succeeding embryo stages.

At first sight the found mitotic indices indicate such differences in cell multiplication within the 18-hour-embryo that would be well consistent with the variations of the quotients in question, but statistical analysis reveals that the figures do not differ significantly (Table I). However, there exists a real difference between part 2 and part 4 in the second of the two investigated 18-hour-embryos.

As for the mitotic spectrum, *i. e.*, the relative distribution of the various phases of mitosis among the dividing cells, it has not been possible either to demonstrate any profound dissimilarities between the parts.

Within the slightly younger embryos (16- and 16—18-hours) reliable differences between mitotic indices are lacking as well, but in the most advanced stages (the 20- and the 23-hour-embryo) the mitotic index of part 1 differs

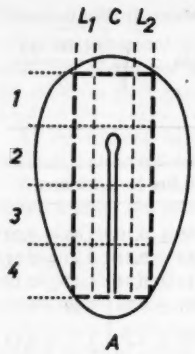

Fig. 1 A. Drawing showing the extension of the regions of the 18-hour-embryo, dealt with in table I. Transversal strips: regions 1—4. Longitudinal strips: regions L_1, C and L_2. Together the regions 1—4 comprise that part of the blastoderm which in the text is designated as embryo area. Embryo explants either constitute the whole of the embryo area or the anterior half thereof (region 1 + anterior half of region 2).
B. Schematic illustration of mitose-accumulation (= striation) in explanted 18-hour-embryos during the first mitotic wave after a series of heat shocks. High degree of accumulation is indicated by dense striation.

noticeably from the other values. This is especially pronounced in the last-mentioned embryo stage, for which the RNA/DNA-quotient of part 1 is actually 50 per cent higher than in parts 2—4.

The general impression from Table I of the primitive streak embryo is that cell multiplication within the embryo area is not as variable as was to be expected from the biochemical data. Notwithstanding, there are strong tendencies towards regional differences just in the suggested direction, and they can scarcely be ignored.

Another important piece of information, recorded in Table I, is that among the investigated stages the average mitotic index for the whole embryo area will reach its highest values in the 18—20-hour-old chick embryo. Earlier findings (EMANUELSSON 1958), have proved that the RNA/DNA-quotient — calculated for the whole embryo area — attains a minimum just in the 20-hour-embryo. Thus for the whole embryo area, at least, the inverse relation between RNA-content and mitotic activity is quite obvious.

In the account given above the estimation of the mitotic activity in the chick embryo has been based entirely upon records of the mitotic index.

Now the generally accepted method of proving the existence of a local cell proliferation in animal tissues is to demonstrate the local occurrence of a significantly higher mitotic index. However, the mitotic index is considered to give the relation between the duration of mitosis and that of interphase, and it is obvious that no direct information is obtained about the generation time of the cells, *i. e.*, time for mitosis + time for interphase (RIS 1955). Consequently it may be difficult to interpret the meaning of an elevated mitotic index correctly. If supplementary information about the investigated tissue is not at hand, *e. g.*, about its normal histologic appearance and the normal duration of morphologic changes etc., it is, paradoxally enough, almost impossible to decide whether an elevated mitotic index is to be regarded as a manifestation of a stimulated or blocked cell multiplication. For further discussions about

the mitotic index, see *e.g.*, WOODARD (1948), HOFFMAN, (1949) and WALKER (1954).

Further, it is easily understood that small differences in mitotic index are definitely proved only after the examination of a vast number of cells.

In the present situation the recorded variations of the mean values of the mitotic index, therefore, seemed worthy of further investigation. It was decided to try to arrest the embryo cells in mitosis and in that way bring about a successive accumulation of mitoses. Then if existent, even minor regional differences in mitotic activity ought to be revealed when comparing the relative accumulation in the various parts of the embryos.

B. *Treatment with Supra-Optimal Temperature*

In the first instance it was hoped to produce at least a limited accumulation of mitoses simply with the aid of a temporarily elevated incubation temperature.

In the ciliate Tetrahymena elevation of the environmental temperature may be so adjusted that the cell (animal) is just completely prevented from entering mitosis, other cell-functions, however, *e. g.*, synthesis of nucleic acids, proceed uninterruptedly (ZEUTHEN and SCHERBAUM 1954). After reversion to the optimal temperature, cell division is started again in the previously blocked cells. As the number of blocked cells gradually increased at the elevated temperature, the result will be that when conditions are favourable for mitosis, a lot of cells will enter this state simultaneously. Thus there will appear a veritable wave of mitoses in the cell culture.

Now conditions are, of course, quite different for chick embryo cells than for Tetrahymena, thus, for instance the former have a much higher optimum temperature. Just owing to this fact it was at first doubtful whether mitosis could be blocked in chick embryo cells by heat treatment and then released en bloc without serious damage to the cells.

KEMP and JUEL (1931) have investigated the heat sensitivity of tissue cultures of the chick heart and report that incubation for 5 min at 47—50° C produced a prolonged, but reversible block of cell multiplication. At 45—46° C the block was less pronounced, whereas 5 min at 42—44° C, finally, was almost without effect upon the mitotic activity. In one single case a just discernible compensatory rise in the mitotic index was observed after the heat treatment.

DEUCHAR (1952) found remarkably good tolerance of primitive streak embryos in ovo towards such an extreme temperature as 45.5° C. Of chick embryos incubated at this temperature for 5 hours in ovo about 50 per cent appeared normal when examined 3—5 days later. However, as no allowance was made for warming of the eggs from room temperature to 45.5° C, the time for the actual heat shock must have been considerably less.

Single Shock

In the beginning of the present experiments, all of which have been made in vitro, embryos at the primitive streak stage were incubated at 45.0° C for

3.5 hours, whereupon incubation was continued for 0—24 hours at 37.5° C. Assuming the generation time of the cells to be at least 8 hours, cf. Ris (1955), the selected time for the treatment was considered adequate for effecting a distinct compensatory wave of mitoses, as the preliminary experiments had revealed that such an effect could be produced.

The embryo cells could just endure this treatment, but if the time was prolonged or if the temperature was raised further there appeared an increased number of pycnotic nuclei as well as pronounced necrosis afterwards.

Embryos examined *immediately after the treatment at 45.0° C* showed slight morphologic changes. However it was apparent in several cases that the primitive streak had broadened considerably. Normal mitotic figures were entirely absent and during the treatment they were observed only immediately after the start of the experiment, when it was obvious that the cells were able to complete such mitoses that already were in progress.

The greater part of the nuclei were after their treatment in a state reminiscent of early prophase. In many cells there had occurred a conversion of the contents of the nucleus into many small heavily stained granula, a phenomenon, commonly observed in embryo cells undergoing mitosis at normal temperature after a previous heat treatment. This arrangement of the nuclear material is considered to correspond to advanced prophase. However, it is assumed that the mitosis process cannot proceed any further at the high temperature, as the nuclear membrane of the cells did not disappear.

Here and there in the preparations a few pycnotic nuclei were to be found, and, in fact, there appeared what may possibly be considered as very much deformed ana- and telophases, but they were very rare.

Staining with Giemsa's solution and with gallocyanin-chromalum revealed that the heat treatment led to a general disappearance of the nucleoli. Together with the observation of a fainter staining of cytoplasm in the treated cells, as compared with controls stained under exactly similar conditions, it indicates that the shock has seriously interfered with RNA-synthesis in the embryos.

During *the following incubation at 37.5° C* embryos were collected at regular intervals and were subjected to morphologic and cytologic examinations.

Even when the inspected embryos had been incubated at normal temperature (37.5°) no less than 24 hours after the heat treatment, normal morphology was not displayed in any case. Generally the primitive streak, when discernible, was much broadened. During the period investigated no embryo exhibited a closed neural groove or distinct brain vesicles. Somites never appeared. Obvious separation of the germ layers along the edges of the explant was noted.

All observations by the author indicate that the normally occurring streaming of the cells in the blastoderm at the primitive streak stage was severely disturbed.

The restoration of normal temperatures soon resulted in the recommencement of mitotic activity. A great many cells that had been prevented from enter-

ing mitosis owing to the inhibiting effect of the high temperature now completed this process simultaneously. As a result during the 24 hours at 37.5° C after the shock the previously comparatively even mitotic activity in the developing embryo was changed into a decided periodicity, reflected in some big waves of mitoses. In fact minor waves between them were also recorded in some cases.

The first big wave comes shortly after the treatment has ceased, and it reaches its maximum about 2 hours after the transfer to the lower temperature. It is to be noted, however, that the different stages of mitosis that are exhibited now during the wave are not of the ordinary appearance.

In prophase distinct chromosome filaments are not to be seen, but instead the nuclei are filled with small granula, more or less distinctly arranged as strings of beads. Later when the nuclear membranes have disappeared and all the dividing cells have entered metaphase, the whole preparation takes on a very strange appearance, crowded as it is with round metaphase-chromosomes, many of which are widely scattered within the cells. Also the anaphase and telophase stages are confusing owing to the abnormally contracted form of the chromosomes. Under the existing circumstances calculation of the mitotic index was almost impossible but during the first wave it was estimated to amount to more than 30 per cent in the region around the anterior part of the primitive streak.

It was evident that the released mitoses were not evenly distributed in the explanted embryos. As far as it could be controlled, the mitotic index was especially high in the vicinity of the primitive streak, and mitoses were obviously more frequent in the ectoderm than in the underlying layers (meso- and entoderm).

The next big wave occurred about 8 hours later, *i. e.*, when the embryos had been incubated for about 10 hours at 37.5° C. As well as during the first wave the prophase and metaphase stages show an abnormal appearance with the chromosome material still in the form of round granula, but at the end of this wave many normal ana- and telophases are distinguished. In many of the anaphases it is obvious that the bivalents have difficulty in accomplishing a separation, hence extremely extended anaphase chromosomes will result.

The third big wave during the investigated period came about 20 hours after the start of incubation at 37.5° C. Even if the embryonic material investigated is still characterized by cells with their chromosome material in the state of granula, the number of normal mitoses is conspicuously large. However, surprisingly many tripolar spindles now appear among the anaphases.

Series of Shocks

In all further experiments in which chick embryos were exposed to high temperature shocks, the author tried to modify the treatment in order to reduce chromosome disturbances in the cells to a minimum without abolishing the

wave-promoting effect. To attain such a result it proved necessary to lower the temperature of the shock and to shorten the duration thereof.

The appropriate temperature appeared to be 44.0° C. At this temperature most embryo cells were still prevented from entering mitosis, but if the temperature was lowered further (*e. g.*, to 43° C) the resulting block was apparently very incomplete. As to the duration it should preferably be about one hour (at 44° C) if big waves of mitoses are to be effected. But even shocks of this length will cause serious disturbances to the embryos, preventing normal morphogenesis.

Eventually a more lenient treatment was arranged so that embryos were exposed to a *series* of heat shocks (44.0° C), alternating with periods when the incubation temperature was lowered to 20° C. A duration of 30 minutes was chosen both for the shocks and for the intervals between them. By keeping the temperature as low as 20° C between the shocks cellular metabolism in the explants was slowed down and the majority of the cells, prevented from entering mitosis during the heat shock, were not able to complete cell-division during the following period of sub-optimal temperature either. Judging from HARRISON and KLEIN's (1954) investigations growth and differentiation in chick blastoderms will proceed at least 30 times more slowly at 20° C than at 37.5° C. However, 20° C is by no means an absolute barrier for cell division. This fact was soon realized in the cytologic control of the explants, and moreover, it was also indicated by the finding that the wave-promoting effect from the treatment was lower than expected. In the lastmentioned type of experiments the treatment usually involved 5—7 × 30 min at the supra-optimal temperature, alternating with 4—6 × 30 min at the sub-optimal temperature and followed by incubation at 37.5° C.

Under such conditions an average mitotic index in the explant of up to 12 per cent could be attained during the following waves of mitoses. In controls the index lay all the time at about 2 per cent.

In Fig. 2 is demonstrated a typical result from an experiment of the type just described. The synchronism which was effected is manifest in the explant still after the completion of two successive cell cycles. In this series and in the others of the same sort the generation-time of the synchronized cells was found to be 12—14 hours, *i. e.*, longer than after a single shock.

Morphogenesis was in no case quite normal during the incubation at optimum temperature after the shocks, but approximately one third of the embryos exhibited less severe disturbances. In the examination of these embryos it was found that the morphologic disturbances had especially affected the outgrowing mesoderm. Development of somites, for instance, was only exceptionally observed. Deformation of the neural tube as well as the primary brain vesicles were frequent.

The distribution of the accumulated mitoses was apparently much similar to that observed in embryos which had been exposed to one single shock. In

addition it was now possible to establish more exact values for the accumulation. Even if individual variations in the average accumulation were considerable in the c. 100 explants investigated, the distributional pattern of the mitoses during the first mitotic wave after the heat shocks was one and the same. Cf. Fig. 1 B.

A representative of these explants is shown in Fig. 4 E. In that very explant — as in the others — the highest average mitotic indices (8—10 per cent) are found in the immediate vicinity of the node and in adjacent areas on both sides thereof. More towards the fore part of the explant the index is somewhat lower (6—7 per cent). Slightly lower values dominate in the hind part of the explant.

A major part of the explants further displayed accumulation of mitoses in a narrow zone along the whole edge of the preparation.

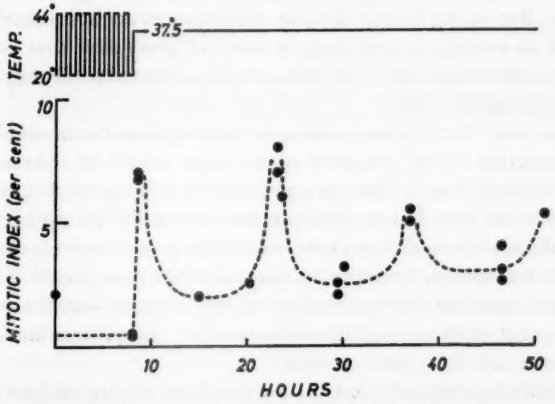

Fig. 2. Diagram illustrating the synchronization which is achieved in explanted chick embryos (18-hour-stage) after a series of heat shocks. Mitotic indices refer to a part of the embryo area, obliquely anterior to the primitive streak. Indices are average values for all cells within the region.

At least for the node region and the area anterior thereto it will seem that the varying accumulation of mitoses rather has its origin in the underlying layers (meso- and entoderm) than in the ectoderm. In the latter layer average accumulation was always strikingly higher than in the two others taken as a whole. In the actual explant, for instance, mitotic indices recorded for the ectoderm amounted to 11—16 per cent against 4—7 per cent for the others. Normal average values for the same stage (in ovo) were found to be: ectoderm 4—6 per cent, mesoderm + entoderm c. 2 per cent.

Summarizing, the experiences of the heat shocks show that it is possible with supra-optimal temperatures to produce waves of mitoses in the chick embryo, *i. e.*, to achieve a partial synchronization of the cell material. However, to

prevent division of the embryo cells the temperature must be considerably elevated above the optimum (37.5° C). RNA-synthesis is apparently very sensitive to change, but probably there is still some margin left before also the DNA-synthesis is affected. Through the compensatory increase of mitoses after a heat shock patterns in cell multiplication, if existent, can be more easily distinguished, and by securing the time for the next wave of mitoses an approximate measure at least of the generation time of the cells is obtained. However, it is apparent that different types of heat shocks give different values of the latter.

C. *Treatment with Mitotic Inhibitors*

Now indications of a higher mitotic activity around the anterior part of the primitive streak and in the ectoderm had been clear enough in the heat shock experiments. But owing to the obvious disturbances in chromosome morphology as well as embryo morphology it was not precluded that the observed distribution of the accumulated mitoses actually reflected a varying heat sensitivity of the blastoderm cells.

It was assumed that further conclusive information of the mitotic activity in the blastoderms of the primitive streak stage would be obtained with the aid of mitotic inhibitors at the normal incubation temperature, 37.5° C. The inhibitors selected have been colchicine and oestradiol. As distinguished from conditions during the heat shocks the inhibition is now in both cases an arrest of mitosis in metaphase. Preliminary investigations had proved that still after 6 hours the beginning disorganization of the arrested mitoses presented no difficulties in the estimation of the mitotic index (or stathmo-kinetic index, as it sometimes is called on such occasions).

Of the inhibitors colchicine, as known, has been widely used for localization of mitotic activity and cell proliferation in animal tissues. It has also been used for chick embryos, and strong indications of considerably differing generation times among the tissues of the 2-day-old chick embryo were found by Overton (1958) after colchicine inhibition.

The inhibiting action of oestradiol on cell-division in vertebrate cells has been described by, among others, v. Möllendorff (1943), (rabbit fibrocytes), and Töndury and Cagianut (1951), (amphibian embryos). Correspondingly inhibition of mitosis in invertebrate cells is described by Agrell (1954, 1955) for sea urchin embryos. The latter author has emphasized the similarity between the inhibiting action of colchicine and that of oestradiol, and has made use of the last-mentioned substance to reveal a graded mitotic activity within the sea urchin embryo, Agrell (1960).

Colchicine

In Fig. 3 are collected mitotic indices for chick blastoderms, incubated for various lengths of time on a medium containing colchicine. The values refer

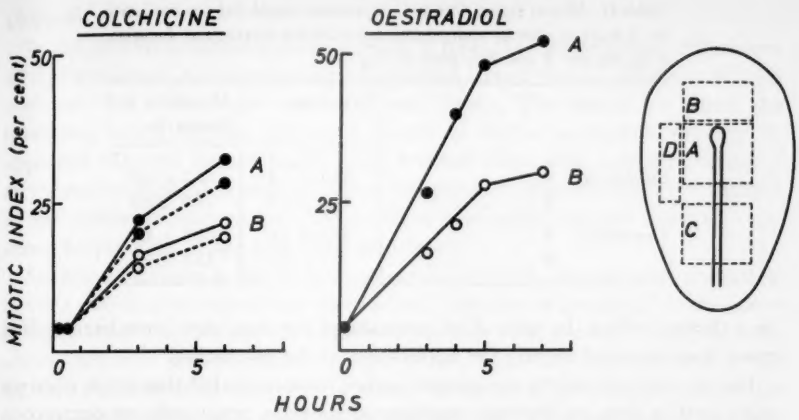

Fig. 3. Diagrams showing the accumulation of mitoses produced by colchicine and oestradiol respectively in 18-hour-old chick embryos. Continuous lines refer to explanted embryos, dashed lines to embryos in ovo. The letters A and B stand for regions marked off in the drawing at right. Each point represents an average from three separate embryos.

to two different regions in the blastoderm, regions A and B, which also are outlined in the figure. They comprise A: the anterior part of the primitive streak and B: the adjacent area in front of the streak. The selection of the particular regions A and B as the principal regions for analysis comes from the fact that they represent adjacent parts of the blastoderm showing the most diverging RNA-concentrations (cf. p. 5), and reasonably ought to be in the best position for showing any differences in mitotic activity, if existent. Values are not given for regions C and D in Fig. 3, as it appeared that they were intermediate between those of A and B, but did not differ significantly from either of them. The reported values are as before mean values for all cells in the delimited region, irrespective of the distribution of the cells among the different germ layers.

As the produced arrest of mitosis was complete, the mitotic figures observed in embryos treated with colchicine for 3 hours and more are almost exclusively metaphases. Of course, there is a small fraction of prophases too, but anaphases and telophases are absent.

The diagram in Fig. 3 demonstrates that all through the investigated period accumulation of mitoses is greater in region A than in region B — both in ovo and in vitro. The difference is significant and has to be taken as an indication of a different mitotic activity in the investigated regions. The distribution of the mitoses is apparently the same as after the heat shocks.

It was surprising to find that accumulation was slightly slower in ovo than in vitro, as according to WADDINGTON (1952) the rates of both growth and differentiation of early chick embryos are slower in vitro than in ovo. This may

Table II. Mitotic indices (per cent) in primitive streak embryos incubated for 3 hours in vitro on media containing colchicine or oestradiol. Location of the regions A and B is given in Fig. 3

		Ectoderm	Mesoderm + Entoderm
Colchicine	A	33.9 ± 1.4	18.1 ± 1.0
	B	31.3 ± 1.4	13.9 ± 0.8
Oestradiol	A	33.5 ± 2.6	21.5 ± 1.3
	B	33.2 ± 2.6	13.2 ± 1.2

be a thermal effect. In spite of all precautions the eggs may have been chilled more than expected during the application of the colchicine.

Earlier observations by the present author have indicated that chick embryos cultivated in vitro on the basic medium alone show practically no decrease in the average mitotic index during the first 10 hours of cultivation. In Fig. 3 it appears, however, that both in ovo and in vitro the accumulation of cells is taking place at a slightly decreasing rate. It is doubtful whether this is to be considered as the normal condition in the embryo tissue, or if it can be ascribed to a general slackening effect from the colchicine. The latter alternative seems the more probable, as it is known that colchicine is toxic in high dosage, LUSHBAUGH (1956). However, the present concentration of colchicine has scarcely been too high as nuclear damage in the treated embryos is insignificant. Besides the same concentration is not toxic for chick embryos of 50—60 hours' incubation (OVERTON 1958).

The morphology of the treated blastoderms was markedly changed after treatment with colchicine. Thus there had occurred a pronounced shrinkage of the area pellucida (as well as the area opaca) during the treatment, resulting in a reduction of the original width of the area pellucida about two thirds after 3 hours' incubation. The shrinking effect from colchicine is manifest also in older chick embryos, causing much distortion of the outgrowing organs. The shrinkage appears to be directly related to changes in cell shape. Investigations by MISZURSKI (1949) seems to indicate that colchicine acts upon viscosity of the cytoplasm in animal cells with the effect that the cells adopt a less extended shape than before.

The estimation of the mitotic index in the investigated regions has been combined with inspections of the distribution of the mitoses in order to check whether they are evenly distributed between the germ layers or show a tendency to local accumulation. It appeared from these examinations that for both the regions A and B accumulation of arrested mitoses is much more rapid in the ectoderm than in mesoderm + entoderm. Cf. Table II. Further the figures intimate that the difference between A and B has little reference to the ectoderm.

Oestradiol

In the present situation it was of interest to have the found regional differences further confirmed in other similar experiments, based upon a different mitotic inhibitor. For this purpose oestradiol was chosen. The author has tested the inhibiting action of pure oestradiol as well as that of oestradiol-benzoate. It appeared that the former had a most decided effect with complete arrest of all mitoses in explanted embryos when present in a concentration 10^{-5}. Added in the corresponding molar concentration oestradiol-benzoate arrested only about 20 per cent of the cells entering mitosis.

Attempts to make a direct comparison of embryos treated with oestradiol in vitro and in ovo respectively have failed. Injection of oestradiol in the same concentration as applied in the experiments in vitro did not lead to pronounced accumulation of metaphases in embryos in ovo. Not even after injection of the fourfold concentration was there a complete arrest of any major part of the cells undergoing mitosis, and further increase of the concentration was not tried, as already the concomitant increase in the alcohol (solvent!) concentration was not quite harmless to the embryo cells.

In essential respects the results from treatment with oestradiol in vitro, Fig. 3, are in agreement with that obtained with colchicine. Also now there is a significant difference in the rate of accumulation of mitoses between the regions A and B, and as before accumulation of arrested mitoses in region C and D is intermediate between that in A and B respectively.

This time accumulation of arrested metaphases is still taking place at an even rate after 5 hours' incubation with the inhibitor. In that period roughly 50 per cent of all cells in A are arrested. Subsequent accumulation proceeds at a slower rate, presumably as the result of a beginning disappearance of the first arrested mitoses. As a matter of fact, it is rather difficult to present exact mitotic counts in chick embryos treated with oestradiol for more than 7—8 hours. At that stage of the treatment the chromosomes in a great part of the arrested mitoses actually have begun to decompose.

From the intensity of the accumulation it may be concluded that the mean generation time of the cells in region A is about 10 hours, while for the cells in region B it amounts to about 15 hours. Corresponding values after colchicine inhibition are of the same magnitude. Table II further informs that for ectoderm cells the generation time is about 8 hours.

The calculated times are in fairly good agreement with values reported for chick embryo cells in tissue culture (Ris 1955). In the literature no information is given about length of generation time in vivo for embryos just at the primitive streak stage, but for embryos of c. 30 hours' incubation calculated values vary between 8.9 hours (Solomon 1957) and 10.6 hours (Woodard 1948).

Localization of the arrested mitoses is the same after treatment with oestradiol as with colchicine, *i. e.*, accumulation of metaphases is most prominent in

the ectoderm cells, decreasing in the mesoderm and still more so in the entoderm. Cf. also Table II.

Oestradiol has no shrinking effect upon the preparation. In fact it has instead a loosening effect upon chick embryo tissues so that blastoderms treated with it have a tendency to flatten out. Even the arrested metaphases do not appear so contracted as during colchicine inhibition.

Discussion

In the present investigation it has accordingly been possible to demonstrate a higher mitotic activity around the anterior part of the primitive streak than in the section of the blastoderm in front of it. The result was strongly indicated already in the heat shock experiments, but was definitely substantiated only with the aid of mitotic inhibitors. On the whole it confirms DERRICK's (1937) observations, though with accentuation of mesoderm and entoderm as the principal sites for the varying mitotic activity within the actual part of the blastoderm. Of these two again, mesoderm has showed greater heterogeneity with respect to the cell multiplication.

However, even now the established difference must be considered as rather moderate, and it will scarcely make the primitive streak stand out as a regular zone of proliferation.

Within the embryo area of the 18-hour-blastoderm there is evidently no region which decisively can be distinguished as such a zone. But if one wishes to emphasize the mutual dependence of cells on different levels in that area, one can, of course, characterize the whole ectoderm as a zone of proliferation in relation to the other germ layers. In investigated normal embryos the relative amount of mitoses was always definitely higher in that layer, and this condition was, of course, still more pronounced in those embryos in which an accumulation of arrested mitoses had been produced.

The fact that the observations to a large extent are based upon embryo explants will not invalidate the obtained result. Indeed growth and differentiation are slightly slower in explanted embryos than in embryos in ovo, but as far as known this retardation will affect all cells equally, and so the explant is still directly equivalent to the normal embryo. Evidence of a continued maintenance of the normal relation between the cells in the embryo area — even after explantation to a basic culture medium of the kind employed here — is given by the fact that such explants will show normal morphogenesis during their further development in vitro.

One complication when comparing mitotic activities in the various regions of the early blastoderm with the aid of mitotic inhibitors is that the primitive streak is no permanent structure but will last only for a comparatively short time. Partly as a result of cell-streaming the streak will soon regress and eventually disappear. For the interpretation of the observations above it is necessary to know whether one can regard a result — obtained through a successive gathering

of cells entering mitosis — as representative for conditions prevailing just at the primitive streak stage. The fact that the difference is significant already after 3 hours, when using two different inhibitors of mitosis, is strongly indicative that such is the case. Judging from the observations of SPRATT (1947), the cell-streaming is not rapid enough at this stage to interfere seriously with the result over such a short period.

The occurrence of definitely higher mitotic indices around the anterior part of the primitive streak, *i. e.*, a section of the blastoderm with a comparatively low value of the quotient RNA/DNA for the stage in question, is reminiscent of conditions in the more advanced chick embryos, investigated by the author. In these embryos (23—40 hours of incubation) regions with the lowest RNA-concentrations are indubitably characterized by a noticeably high mitotic activity.

It is doubtful, however, whether the connection observed in the 18-hour-blastoderm is fully equivalent to that prevailing later in development in the outgrowing embryo body. For in view of the obvious differences in RNA-concentration between the investigated regions more striking contrasts in mitotic activity were indeed to be expected. Thus it will seem that of the observed RNA-variation at this stage, a good deal at least cannot be attributed to a non-uniform cell multiplication within the blastoderm. Rather it will reflect the differentiation, taking place within the blastoderm.

As each of the selected pieces of the embryo area (1—4 in Fig. 1) reaches right through the blastoderm there is, by the very presence of outgrowing mesoderm in all of them, a general differentiation going on in the whole area. The particularly high concentration of RNA in the region immediately in front of the node may probably be ascribed to the intense protein synthesis during the beginning differentiation of the notochord and neural plate cells. Perhaps a certain accumulation of RNA will also be connected with the onset of induction within the region in question, as according to BRACHET (1957) transfer of RNA is probably involved in that process.

As a matter of fact, histochemical analyses of the present author for RNA (based upon examination of RNAse-treated embryos and controls, both stained with gallocyanin-chromalum) as well as those of GALLERA and OPRECHT (1948) and LAVARACK (1957) have just indicated that in front of the node mesodermal cells, and still more so ectodermal cells, are very rich in RNA. Even if the cells of the primitive streak obviously have a high content of RNA too, the visual estimation of RNA seems to be consistent with the result of the biochemical analyses, *i. e.*, of a higher average value of RNA for the region immediately in front of the streak.

In this connection the regional differences in carbohydrate metabolism reported to occur in chick blastoderms are worth mentioning. According to SPRATT (1950) the anterior part of the primitive streak (the node) has a conspicuously higher energy requirement of carbohydrate than other regions, whether dif-

ferentiated or undifferentiated within the blastoderm, even if it has not yet been possible to prove this in respiratory measurements. The higher mitotic activity in the node region found in the present investigation seems well consistent with SPRATT's observation. The same author also distinguishes a qualitative difference in carbohydrate metabolism between the heart mesoderm and the neural ectoderm, growing out in front of the node. Thus the latter would depend primarily upon oxidative metabolism, the latter upon glycolysis; however, this interpretation has been seriously questioned by DUFFEY and EBERT (1957).

In the present investigation it has been of great interest to follow *the morphogenetic and cytologic disturbances* which have been provoked in the blastoderm by heat and by mitotic inhibitors.

It was not surprising to find that heat treatment affects the synthesis of RNA in the chick embryo; a similar result is reported for amphibian embryos, STEINERT (1951). As RNA-protein and RNA presumably play an important role in inductive processes in the developing embryo and as furthermore it has been argued that RNA will contribute to a proper cell contact (BRACHET 1957), it seems that there is a direct connection between this heat effect upon RNA and the recorded morphogenetic disturbances.

When analyzing the influence of elevated incubation temperatures on dividing chick embryo cells, one should try to distinguish between effects on DNA-synthesis and effects on the mechanism of division, *i. e.*, on processes connected with the movements of the chromosomes. Apparently the mitotic disturbances produced in short-time experiments by extreme temperatures, high as well as low, belong to the last-mentioned category, (KEMP and JUEL 1931, SPEAR 1928). Thus it is also presumed in the present investigation that the compensatory increase of mitotic activity after a heat shock (the mitotic wave) will indicate that mitosis, but not DNA-synthesis, was brought to a standstill during the shock. As in the present case the cells were observed to be arrested in prophase, it seems likely that the heat shock has affected such processes as chromosome spiralization, migration of the centrioles and aster formation.

In this connection attention should again be given to the above-mentioned decrease of the RNA-content of the heat-shocked cells.

Evidence for a relation between ribonucleoproteins and the chromosome cycle has been presented by JACOBSON and WEBB (1952) and according to GUTTMAN (1956) the chromosomal ribonucleoprotein possibly controls chromosomal spiralization and despiralization. Experimental removal (by RNAse) of cellular RNA in chick fibroblasts, has in fact been found to result in prophasic inhibition of mitosis (FIRKET *et al.* 1955). In meristematic cells, multipolar spindles among other abnormalities were found after influence of the same enzyme (KAUFMAN and DAS 1955).

Thus it appears that a disturbed RNA-synthesis may actually account for both the morphologic and chromosomal disturbances, observed after the heat shocks.

The morphogenetic disturbances in chick embryos, caused by oestradiol are similar to those effected by heat treatment in the respect that the mesoderm is especially affected. Blastoderms explanted at the primitive streak stage to a culture medium, containing a low concentration of oestradiol (2.6 μg/ml medium) show no morphogenesis after 24 hours' incubation. When explanted at the 20-hour-stage, one observes after the same time a neutral tube and brain vesicles, but heart and somites are missing. If explants of the primitive streak stage are first incubated for half an hour on a medium containing oestradiol (2.6 μg/ml medium) and then are transferred to a normal medium, they will show a slight retardation only in the development of heart and somites. The disturbances will possibly depend on interference with the synthesis of RNA. For the closely related substance stilboestrol there are strong indications of just a blocking effect upon RNA-synthesis in chick embryo cells (RICKENBACHER 1956). In Triton embryos a diminished RNA-content in the cells is found after treatment with oestradiol (TÖNDURY and CAGIANUT 1951).

The rectilinear accumulation of arrested mitoses in the oestradiol-treated chick embryos indicates that there is no blockage of DNA-synthesis during the interval of the inhibition experiments. It is quite a different matter that after prolonged treatment at high oestradiol-concentrations signs of degeneration are observed in the arrested cells, the destruction being accompanied by a transformation of the chromosome material into one or more intensely stained droplets. Experiments in vitro have shown, however, that oestradiol can act as a substrate activator for DNAse by separating the proteins from DNA (AGRELL and PERSSON 1956).

By choosing a sufficiently high concentration of oestradiol in the experiments a total arrest of all mitoses was secured, and the apparent differences in sensitivity of the germ layers towards the concentration of oestradiol, as observed in explants, could be disregarded. The fact that a similar distribution of arrested mitoses between germ layers is found after colchicine inhibition, cf. Table II, is taken as an indication that oestradiol has not specifically changed mitotic activity of any particular germ layer within the investigated region.

Neither is it likely that the established difference in cell multiplication between region A and region B reflects a qualitatively different reaction towards oestradiol. Actually all observations intimate that the regional differences established by means of oestradiol are directly equivalent to those demonstrated in the colchicine experiments.

The real striking difference in action between oestradiol and colchicine is the dissimilar ability of mitose-accumulation that they show when the embryo material has been exposed to them for more than 3 hours. In the experiments the rate of accumulation will thus decrease in the colchicine material, whereas it proceeds at the earlier rate in oestradiol-treated explants. As already suggested, this decreasing rate observed during treatment with colchicine is probably effected by the colchicine itself. For as this inhibitor produces the same

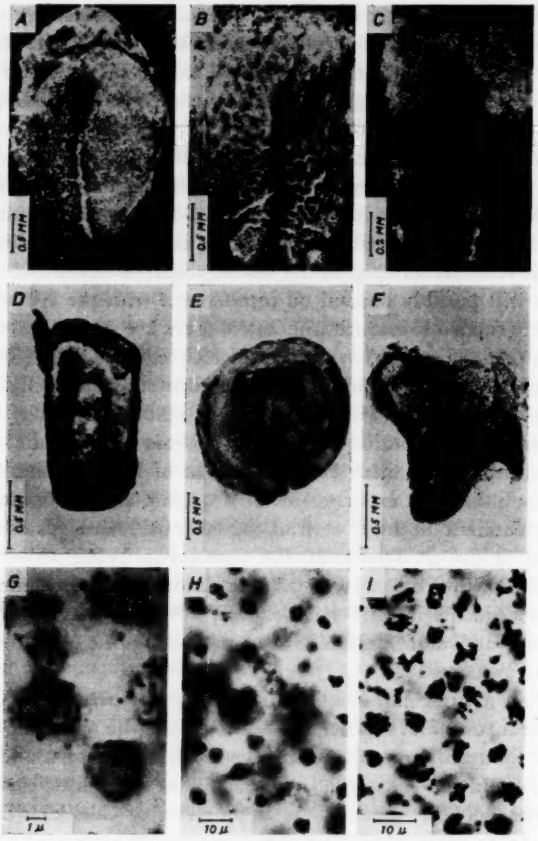

Fig. 4 A. Normal chick embryo at the primitive streak stage (18 hours of incubation).
B. Chick embryo at the primitive streak stage incubated with colchicine in ovo for 3 hours. Notice the mottled appearance, characteristic for embryos exposed to colchicine in experiments of short duration.
C. Chick embryo at the primitive streak stage incubated with colchicine in ovo for 6 hours. The extreme shrinkage of the embryo is very apparent. (Notice the different magnification in A, B and C.)
D. Explanted chick embryo (anterior half of the embryo area) immediately after a series of heat shocks. Notice the broadened primitive streak.
E. Explanted chick embryo (anterior half of the embryo area), exposed to a series of heat shocks and then incubated for 10 hours at 37.5° C. A developing neural tube is vaguely discerned in the midline of the explant. Incubation was interrupted at the culmination of the second mitotic wave.
F. Explanted chick embryo (anterior half of the embryo area), exposed to a series of heat shocks and then incubated for 20 hours at 37.5° C. Notice the disturbed outgrowth of the neural tube and the absence of somites.
G. Characteristic appearance of prophases of chick embryo cells during incubation at normal temperature (37.5° C) after previous heat shock.
H. Chick embryo cells, arrested in metaphase by colchicine.
I. Chick embryo cells, arrested in metaphase by oestradiol.
Staining: D: Giemsa, otherwise Gomori hematoxylin.

accumulation of mitoses in vivo as well as in vitro the decrease does not reflect a mitotic pattern particularly characteristic of explants. Neither is there discernible any stimulating action on the cells from the oestradiol: in fact both inhibitors have during the first 3-hour-period given practically the same rate of accumulation and it is only just maintained in the explants treated with oestradiol. Otherwise it has been demonstrated that oestradiol can have a stimulating action on mitotic activity (BULLOUGH 1955).

In view of these observations it seems that oestradiol is preferable to colchicine for determination of cellular doubling times in chick embryo explants. In experiments on chick embryos in ovo colchicine has been the only possible of the two, but care must be taken that the produced accumulation is recorded not later than 3 hours after the onset of the experiment.

The shrinkage of the embryo area after treatment with colchicine emphasizes the fact that besides cell multiplication and cell streaming also changes in cell shape play an important role in accomplishment of a normal morphogenesis. Differences in cell shape are very apparent at the primitive streak stage, the extremities are the tall columnar cells in the ectoderm of the streak region and the flat endodermal cells. Moreover, SPRATT (1956, 1958) has recently called attention to the existence in the intact blastoderm of distinct intercellular fibres, which apparently are of great importance for the proper contact between the embryo cells.

The present observations on chick embryos, treated in ovo with colchicine for 1—3 hours (Fig. 4 B) suggest an arrangement within the area pellucida of distinct points with particularly intense adhesion between the cells. It seems that colchicine may be a useful tool for estimation of the quantitative distribution of the above-mentioned intercellular filaments.

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Effect of Intravenous Infusion of Glucose on Gastric Secretory Responses to Feeding in Pavlov- and Heidenhain-pouch Dogs

By

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Abstract

Dotevall, G. and A. Muren. *Effect of intravenous infusion of glucose on gastric secretory response to feeding in Pavlov- and Heidenhain-pouch dogs.* Acta physiol. scand. 1961. *52.* 234—241. — Glucose solution was infused intravenously (30 %, 80—100 ml, 30 min) in conscious dogs with Pavlov- or Heidenhain-pouches during secretion elicited by different procedures. In Pavlov-pouch dogs a pronounced inhibition of the secretory response was observed during sham-feeding or teasing with food; the response to feeding was also temporarily depressed, at least during the first two hours after feeding. In Heidenhain-dogs the response to feeding compared with the Pavlov-dogs was not significantly affected except during special conditions. It is suggested that the induced hyperglycemia mainly depresses the nervous phase of secretion, possibly both at the central and the peripheral level.

The stimulating effect of hypoglycemia on gastric secretion has been extensively studied and the insulin test is commonly used as a way of judging whether the vagal innervation is intact or not. The effect of hyperglycemia on gastric secretion, on the other hand, has not attracted much attention. There are, however, some reports on an inhibitory effect on secretion obtained by acute administration of glucose (Okada *et al.* 1934, Noble and Robertson 1938, Day and Komarov 1939, Friedman 1939, Solomon and Spiro 1959). In most of these cases very large doses of glucose were given. The mechanism of the inhibition has not been further analysed, but Babkin (1950) has discussed the different possibilities and he suggests that hyperglycemia induced by intra-

venous administration of glucose may lower the irritability of the vagal secretory center in the brain, just as hypoglycemia raises it. Others believe that the secretory inhibition caused by hyperglycemia is largely a peripheral osmotic effect (NOBLE and ROBERTSON, DAY and KOMAROV, FRIEDMAN).

In patients with diabetes mellitus, a more or less permanent hyperglycemia is often observed, in spite of treatment with insulin. It has been found that the incidence of duodenal ulcer as well as the gastric secretory activity is reduced in patients with diabetes mellitus (for literature see DOTEVALL 1959, 1961). Furthermore there seems to be an inverse relationship between the degree of hyperglycemia and the secretory capacity of the stomach (DOTEVALL 1961). Experiments on dogs made diabetic by treatment with alloxan or by pancreatectomy (MAYO *et al.* 1958, BARCENA *et al.* 1956, SOELZER 1960) have, on the other hand, given results which are not in accordance with those mentioned above. Such experiments do, however, involve so complex changes with influences on different mechanisms that it is difficult to draw any conclusions from these results, — these reservations should be borne in mind even in the cases of human diabetes. There are thus reasons for believing that hyperglycemia may directly or indirectly, at least to some extent be responsible for changes in gastric secretory activity.

The present paper is concerned with the effect of hyperglycemia on gastric secretion after feeding and other stimulating procedures in Pavlov- and Heidenhain-pouch dogs. A preliminary report of the result has been given previously (DOTEVALL and MUREN 1960).

Material and methods

The experiments were made on 10 dogs weighing 12—15 kg. Pavlov- or Heidenhain-pouches had previously been made; in some dogs the Pavlov-pouch was changed into a Heidenhain-pouch after a period of time so that experiments were made on the same dog with both types of pouches. Oesophageal fistulae were made on two dogs.

Feeding was controlled throughout the experiments. The dogs were given water and a standard dog food (Bonzo) consisting of ground and dried meat, fish and bread formed into small cubes. The experiments were performed on conscious dogs who had been fasted during 24 hours. Before the test meal was given, consisting of 200 g of this food, a needle was introduced into a vein of one hind leg. The needle was connected to an infusion pump via a polythene tube. Infusion of 30 % glucose solution was given usually during 30 min and at a rate of 3 ml/min (*i. e.* a total of 2.5 g glucose per kg body weight). The gastric juice was collected in 15 min periods. The amount of total acid was determined by titrating each portion against N/10 NaOH with phenolphtalein as an indicator.

Blood sugar was determined in some of the experiments according to Somogyi-Nelson's method (NELSON 1944).

Results

1. *Pavlov-pouch dogs.* Nine experiments were made on four dogs. Glucose infusion was started 30—90 min after feeding or during the last part of the rising phase of the secretion curve. In all experiments this caused a distinct depression of

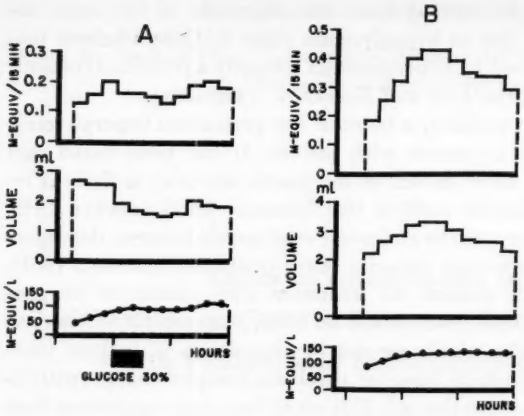


Fig. 1. A. Mean of all observations with glucose infusion after feeding in Pavlov-pouch dogs.

B. Mean of all control experiments after feeding in four Pavlov-pouch dogs.

the secretion, measured in milliequivalents per period; in most cases both volume and acidity were reduced. The effect of infusion was considered an inhibitory one, when the last sample during infusion and at least the two following ones had lower values than the one preceding and two succeeding samples. The mean of all these observations is given in Fig. 1 A. The mean of 7 control experiments after feeding only is given in Fig. 1 B. Fig. 2 presents a comparison of the effects in one and the same dog with high secretion of 30 % glucose, isotonic glucose and the response to feeding only. Fig. 5 A presents the effect of 30 % glucose in a dog with very low secretion. In most cases the effect of the glucose on secretion did not become noticeable until 15 min after starting the infusion. The maximal depression occurred during the first or second period after infusion. The secretion was never completely abolished,

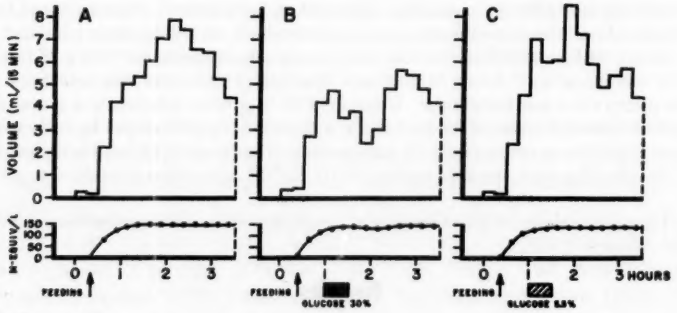


Fig. 2. Three experiments on one and the same Pavlov-pouch dog. (2—3 weeks between each exp.)
A. Feeding only. B. Feeding with infusion of 30 % glucose. C. Feeding with infusion of isotonic glucose.

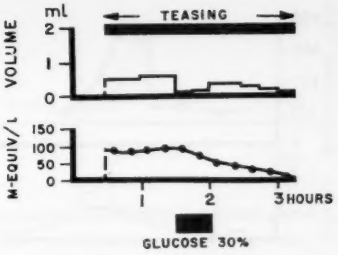


Fig. 3. Glucose infusion during teasing in a Pavlov-pouch dog.

only more or less reduced, usually for 45 min after the end of infusion. The infusion of 30 % glucose caused a bloodsugar increase up to 300—400 mg %, whereas with isotonic glucose solution, the level of bloodsugar did not exceed 150 mg %, which had little or no effect on gastric secretion.

A general observation made during the experiments was that the inhibitory effect of glucose was most pronounced during the early phase of secretion. When glucose was administered more than two hours after the onset of secretion, its inhibitory effect was insignificant or it did not appear at all.

2. *Sham-feeding or teasing.* These procedures, which were tried in 6 experiments on 5 dogs, gave rise to a relatively small and somewhat variable secretion; but the reaction to glucose was very striking. The infusion of 30 % glucose solution caused a rapid and pronounced depression of the secretion (Fig. 3). In most cases the secretion was practically abolished during quite a long period of time after infusion.

3. *Heidenhain-pouch dogs.* In 13 experiments on 4 different dogs glucose was infused during secretion after feeding using the method as previously described for Pavlov-pouch dogs. In these cases the secretory response to feeding was, as might be expected, smaller. In 7 out of these experiments no effect on secretion could be observed (Fig. 4, 5 B). In 4 experiments a minute lowering of volume was seen 45 to 60 min after the end of glucose infusion, in one of those also a slight lowering of acidity. In extent these effects were however so small that they in no way could be compared with those regularly found in the Pavlov-pouch dogs. In the remaining 2 experiments a slight but definite inhibition was observed; however, in these cases there was a basal secretion of acid before feeding. This secretion was not due to an incomplete fasting, but the experiments were made only 3 weeks after changing the previous Pavlov-pouch into a Heidenhain-pouch, a procedure which sometimes has been found to give rise to a basal secretion for which the cause is so far not known.

Control experiments: a) Insulin, 0.5 IU/kg subcutaneously, was given in 8 experiments on Pavlov-pouch dogs. The secretion started within one hour. Infusion of 30 % glucose brought about an abolition of the secretion which lasted for about one hour (Fig. 6). There was a good correspondence between the bloodsugar level and the secretory rate. The fall in secretion started within

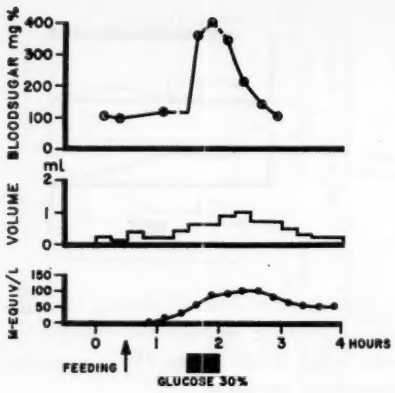
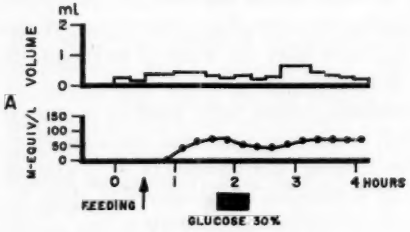


Fig. 4. Glucose infusion during secretion after feeding in a Heidenhain-pouch dog.



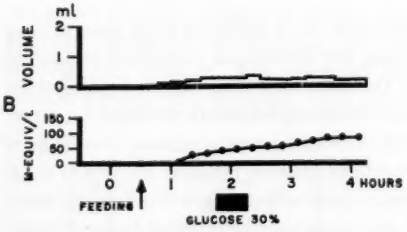


Fig. 5. Glucose infusion during secretion after feeding in one and the same dog.

A. The dog is equipped with a Pavlov-pouch.

B. Three months after changing the Pavlov-pouch into a Heidenhain-pouch.

the first minutes of the infusion, during which the bloodsugar increased up to 300—400 mg %. During the falling phase of the bloodsugar level the secretion did not reappear until the level of blood sugar had reached 60—50 mg %.

b) Saline solution, isotonic (0.9 %) as well as hypertonic (5.0 %) was infused in several experiments during a secretion phase caused by insulin or by feeding. No effects of these infusions were observed, neither on volume nor acidity (Fig. 7).

c) Isotonic glucose solution did, as previously mentioned, not significantly influence the secretion, but in the amounts given it raised the bloodsugar level only to about 150 mg %.

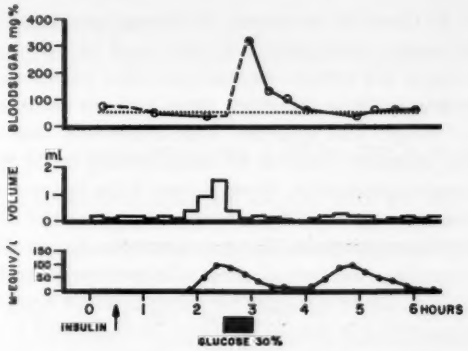


Fig. 6. Effect of glucose infusion on the secretory response to insulin.

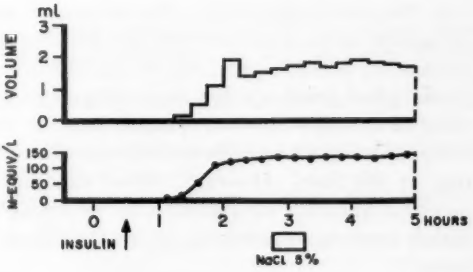


Fig. 7. Infusion of hypertonic saline solution during insulin stimulated secretion.

Discussion

The present results confirm previous findings of an inhibitory effect on gastric secretion caused by intravenously administered glucose. As for the mechanism responsible for this inhibition, the results indicate that hyperglycemia mainly affects the nervous phase of secretion. A peripheral, inhibitory effect due to the increase in osmotic pressure of the blood may be ruled out in the present experiments as saline solutions of the same osmolarity as the glucose solution used did not affect any type of secretion. This is not in accordance with the opinion of the previously mentioned authors, who have made the increase in osmotic pressure responsible for the peripheral inhibition of secretion. This discrepancy may very well be explained by the differences in dosage. In most previous studies large doses of glucose were given, or the solutions were injected intravenously during a short period of time, as compared with the slow, intravenous infusions performed in the present experiments.

Even if the effect of hyperglycemia may be essentially restricted to the nervous phase of secretion there are still a number of different locations and ways through which the effect may operate.

a) Cerebral structures, including cortical areas, vagal secretory centers and synaptic connections may very well be affected at different levels. Since the dogs in the present experiments were conscious, it is possible that the induced hyperglycemia depresses vagal activity by temporarily abolishing the feeling of hunger and appetite. This could very well explain the rapid inhibition of the secretion induced by sham-feeding or by teasing. As for the different subcortical structures, there is very little information at present available, but it has been assumed by several authors that the vagal secretory center is inhibited by hyperglycemia. The present studies do not allow any conclusion to be drawn regarding the central effect of hyperglycemia. It is possible that some information could be obtained by using different types and levels of anesthesia. In this connection it should be borne in mind that a possible, central inhibition may be due either to a depression of the basal activity of vagal centers or by activation of inhibitory fibres, the existence of which is yet uncertain.

b) Peripheral synapses, *i. e.* the connection between central vagal fibres and intramural neurons and between the different neurons constituting the nervous plexa in the stomach walls, may be inhibited by hyperglycemia. From the neurophysiological point of view, no investigations seem to have been made on the effect of hyperglycemia on synaptic impulse transmission. The present results do not offer any direct evidence in favour of an inhibitory effect of glucose operating at this level. However, recent experiments, partly on the same dogs, suggest that such a mechanism may be engaged, as the effect of carbachol, mainly exerting its action on the synaptic level, is also inhibited by glucose infusion.

c) Nerve endings or neuroeffector junctions may be influenced by glucose. The above mentioned recent study indicates that intravenous infusion of glucose to some extent inhibits the secretory responses not only to carbachol but also to mecholyl and urecholine while the histamine effect is quite unaffected (DOTEVALL and MUREN 1961). This may mean that glucose selectively interferes also with the stimulation process at this level. It is generally assumed that histamine exerts its action very close to the secretory cell. However, since so little is known about the physiological mechanism operating at this point no further conclusions can at present be drawn about these problems.

It may be concluded from the present results that acutely induced hyperglycemia can inhibit gastric secretion. There is reason to believe that it is mainly the nervous phase which is influenced and the effect may, at least in part, be localized at a peripheral level. The experiments give no evidence in favour of the assumption that the increase in osmotic pressure induced by the glucose administered inhibits the activity of the secretory cells directly.

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Effects of Ethanol on the Membrane Potential and Membrane Resistance of Frog Muscle Fibres

By

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Abstract

KNUTSSON, E., *Effects of ethanol on the membrane potential and membrane resistance of frog muscle fibres.* Acta physiol. scand. 1961. *52.* 242—253. — The effects of ethanol on the membrane potential and membrane resistance of frog muscle fibres *in vitro* were studied by means of intracellular microelectrode technique. By continuous recording of the membrane potential level from one fibre, as well as by consecutive recordings from different fibres, the time course of the potential changes induced by different concentrations of ethanol was followed for periods of up to one hour. At the lowest concentrations used, 0.05 and 0.1 M, a depolarization, although slow and sometimes irregular, could usually be demonstrated. At a concentration equal to or higher than 0.2 M, a marked lowering of the membrane potential value was regularly observed. The depolarization rate increased with higher ethanol content, a typical result being a 15 per cent reduction of the initial membrane potential value after 15 min of exposure to 1.0 M ethanol. Reversal of the membrane potential changes after the ethanol had been washed out from the muscle bath was also demonstrated. Determinations of the voltage-current relation of the muscle fibre, either by square pulse analysis or by direct recording of voltage-current curves, revealed a gradual lowering of the membrane resistance, the effect increasing with the ethanol concentration from 0.2 to 1.0 M; at lower concentrations the changes in membrane resistance were within the normal variations found in untreated muscle.

One of the earliest observations on excitability changes induced by ethanol was made by VON HUMBOLDT (1797) who on frog nerve-muscle preparations found an initial increase in excitability followed by a decrease leading to final

block. Later BIEDERMANN (1881), by measuring the thresholds for electrical stimulation of motor nerve exposed to ethanol, demonstrated an initial decrease in threshold followed by an increase, and similar excitability changes were also found in experiments with direct stimulation of frog muscle exposed to ethanol (BLUMENTHAL 1896).

While numerous studies of the effects of ethanol on the excitability of nerve and muscle tissue have since been published (cf. *e. g.* LUCAS 1913, BLUME 1925, KOCHMANN 1936), there seems to be but little work bearing on the fundamental membrane processes involved. In a study on frog sciatic nerve, GALLEGO (1948) showed that ethanol applied to a nerve segment caused demarcation potentials indicating a depolarization of the nerve membranes in the affected region. The lowering of the membrane potential was considered as a sufficient explanation for both the increase and decrease in excitability, the latter appearing as a result of cathodal depression at higher degrees of depolarization.

The intracellular microelectrode technique, although applied in numerous studies of other drugs, has apparently not been used in any systematic analysis of the mechanism of ethanol action on excitable membranes. The present paper gives an account of investigations designed to study, with this technique, both the membrane potential level and the membrane resistance in frog muscle preparations exposed to ethanol in different concentrations.

Methods

Preparation. The experiments were done on sartorius muscles of frog (*Rana temporaria*) which were dissected out and placed in a bath containing Ringer's solution of the following composition (mM), *viz.* NaCl 115, KCl 2.5, $CaCl_2$ 1.8, Na_2HPO_4 2.15, NaH_2PO_4 0.85 and with a pH value between 7.0 and 7.5. The whole bath was enclosed in a gas chamber of perspex through which 100 per cent oxygen was passed so as to obtain a constant oxygenation of the bath. In some experiments the bath was oxygenated by bubbling of oxygen through the Ringer's solution. For the purpose of studying ethanol action under anoxic conditions, the oxygen could be replaced by nitrogen.

Ethanol was added to the Ringer's solution in the bath. In some experiments the frog's second sartorius muscle was used as control; it was then placed in a separate bath and treated in the same way as the test muscle except that no ethanol was administered. All experiments were performed at room temperatures between 22 and 25° C.

Microelectrodes. The capillary electrodes (tip diameter less than 1 μ) were produced, by means of a pulling device, from Pyrex glass tubes of about 0.8 mm outer and about 0.5 mm inner diameter. The micropipettes were filled with 2.7 M KCl solution by boiling in ethanol at reduced pressure (cf. TASAKI *et al.* 1954). The resistance of the microelectrodes was checked by application of alternating current of constant amplitude across the electrode from a variable frequency oscillator (cf. HAAPANEN *et al.* 1958). For the experiments were chosen electrodes with resistances between 12 and 30 megohms.

Variations in the tip junction potentials of the micropipettes may introduce a certain inaccuracy in the determination of the numeric value of the membrane potential (cf. ADRIAN 1956). To reduce this source of error, electrodes with relatively low tip potentials should preferably be used. The tip junction potentials have been measured in the follow-

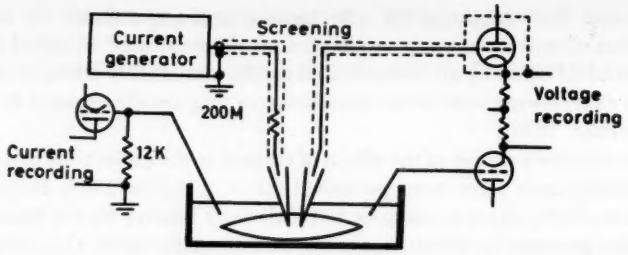

Fig. 1. Diagram of experimental arrangement for determination of voltage-current relation.

ing way. The micropipette was first placed with the tip in 2.7 M KCl solution. This solution being of the same composition as the electrolyte within the capillary, no potential should develop across the tip junction. The electrode was then transferred to Ringer's solution and the change in potential level was measured. This corresponds to the tip junction potential, the potential changes at the reference agar-Ringer electrode being negligible. For the electrodes used in the present investigation the tip junction potentials were found to be up to 8 mV. The administration to the Ringer's solution of ethanol in the concentrations used in these experiments did not result in any recordable changes of the tip potential.

Membrane potential recording. The microelectrode was connected to an input stage of cathode follower type (HAAPANEN and OTTOSON 1954) coupled to a dc amplifier (HAAPANEN 1953) driving one channel of a double-beam oscilloscope as well as an inkwriter (frequency response 1/sec).

Two different types of experiment were used for the studies of the membrane potential changes. In the first, the resting potential of one fibre was recorded continuously for a comparatively long period, up to about an hour. For such long-lasting recordings a baseline drift has to be anticipated, mainly due to diffusion of ions at the site of the microelectrode (dc drift in the amplifier, about 0.5 mV per hour, is negligible, see HAAPANEN 1953). The baseline drift was compensated for by measuring the membrane potentials on the inkwriter curve from the baseline obtained by connecting the zero levels registered by the microelectrode in Ringer's solution before and after the muscle recording. In the second type of experiment, consecutive brief recordings (up to one minute) were made of the membrane potentials of a number of different fibres in the muscle before and after administration of ethanol, in some cases also after the ethanol had been washed out.

Determination of voltage-current relation. By inserting two microelectrodes into the same cell, using one to pass an electric current through the cell and the other to record the resultant potential deflexion, it is possible to study the voltage-current relation, for determination of the membrane resistance (cf. FATT and KATZ 1951). The arrangement for the present investigation is shown in Fig. 1. The electrodes were kept less than 0.1 mm apart, except in a few experiments where the interelectrode distance was 0.4 mm.

Two different procedures were used. In the first, current was applied as hyperpolarizing square pulses of 50—100 msec duration, given at about two-minute intervals. The applied current was recorded as a potential drop across a known resistance on one beam of the oscilloscope and the resultant electrotonic potential on the other beam. The voltage-current relation (V: I) was determined when the electrotonic potential had reached a steady level. Ethanol was not added to the bath until several consecutive recordings had shown a reasonably constant V: I ratio.

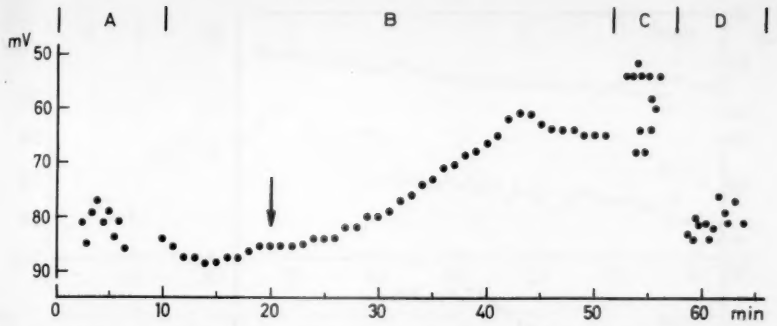

Fig. 2. Ethanol effects on membrane potentials of frog sartorius fibres measured in continuous recording from one fibre (*B*). Ethanol added at arrow, concentration 1.0 M. Curve based on values measured from inkwriter curve at one-minute intervals. *A* and *C* control measurements from different fibres of same muscle at beginning and end of experiment. *D*, comparative measurements on other sartorius muscle of same frog, not exposed to ethanol. Abscissae: time in minutes from onset of recording. Ordinates: membrane potential values (minus sign omitted).

In the second procedure, the X-Y display feature of the oscilloscope (Tektronix 502) was used and variations of applied current intensity recorded as vertical deflexions and concomitant changes in membrane potential as horizontal deflexions of the oscilloscope beam. The current generator was operated manually so as to give gradually increasing current from 0.2—0.4 μA inward to 0.1—0.3 μA outward current; thus a direct recording of a voltage-current curve was obtained (cf. FURSHPAN and POTTER 1959, HUTTER and PADSHA 1959). The rate of the gradual increase could be kept rather constant, the average time used for each of these recordings being about 1 sec. For analysis the oscilloscope trace was photographed and its image enlarged and traced onto coordinate papers.

Results

I. Changes in membrane potential level

Typical results from an experiment with continuous membrane potential measurement are illustrated in Fig. 2. The two sartorius muscles of a frog were placed in separate baths, one of them for exposure to ethanol, the other serving as control. First about 10 fibres were measured in order to gain an idea of the average value of the membrane potentials (*A*), the range of which was found to be from 77 to 86 mV, with a mean value of 82 mV. Then one fibre (*B*), with a membrane potential value in the upper part of this range (84 mV), was selected for the continuous recording. During the 10 minutes of recording before ethanol was administered the potential varied between 84 and 89 mV; at the moment of administration it was 86 mV.

After ethanol had been added to the bath, to a concentration of 1.0 M, a gradual depolarization of the membrane could be observed, in this case at a slow rate during the first minutes of ethanol exposure and faster after the 6th minute. In the 22nd to 25th minutes maximum depolarization was reached,

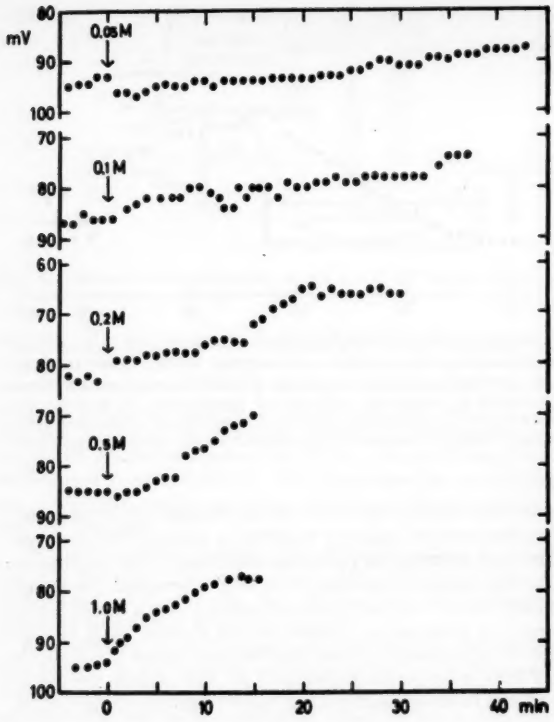

Fig. 3. Continuous recordings of membrane potentials from muscle fibres exposed to ethanol (at arrows) in concentrations of 0.05, 0.1, 0.2, 0.5 and 1.0 M. Curves obtained from different muscles and based on values measured from inkwriter curve at one-minute intervals. Time in minutes from moment of ethanol administration.

and in the last minutes of the recording the level was comparatively even, the final value after 30 minutes of ethanol exposure being 65 mV.

After this continuous recording from one fibre, the membrane potentials of a number of other fibres were also studied (Fig. 2 *C*). Measurements of twelve fibres — thus tested after being exposed to ethanol for 33—36 min — gave a mean value of 58 mV, range from 52 to 68 mV. Fibre membranes not exposed to long-lasting microelectrode injury were thus depolarized to about the same extent as a fibre membrane during continuous recording. Measurements from the other sartorius muscle of the same frog (*D*) gave a mean value of 82 mV, range from 76 to 84 mV. The conformity thus found between the records in *A* and *D* indicates that there has been no significant change in the recording conditions nor in the state of the untreated muscle fibre.

Although there may be some variations in different experiments, the depolarization course shown in Fig. 2 is characteristic of the effects of ethanol at the concentration 1.0 M in the presence of oxygen. To some extent, the depolarization seems to depend on the supply of oxygen in the bath, the depolarization

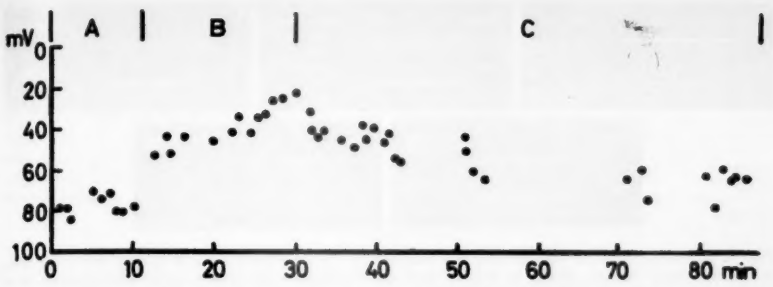

Fig. 4. Ethanol effects on membrane potentials as measured in recordings from different fibres chosen at random. *A* before, *B* after administration of ethanol (concentration 1.2 M), *C* after removal of ethanol. Time in minutes from onset of recording.

rate being considerably slowed down under conditions of anoxia. The difference thus observed may however have a complex background, and it has been deemed necessary to limit the present investigation to experiments with oxygenated Ringer's solution.

Continuous recordings of the membrane potentials have been performed on 22 muscles. Fig. 3 illustrates five recordings of this type from experiments at different ethanol concentrations from 0.05 to 1.0 M. At the lowest concentration the depolarization developed very slowly, amounting to only 6 mV after 43 min of ethanol exposure. At the higher concentrations applied, the depolarization rate increased with the ethanol content, reaching 17 mV in 15 min at a concentration of 1.0 M. It was fairly characteristic that the depolarization at lower concentrations had an uneven course with fluctuations and "plateaus", whereas at higher concentrations the course was smoother.

A definite depolarization could always be observed when the ethanol concentration was equal to or higher than 0.2 M. At the lower concentrations, 0.05 and 0.1 M, slow depolarizations were usually observed — as shown in the figure — but there have also been cases when no change of the membrane potential was perceptible, as also cases of transient hyperpolarization. These variations may possibly be due to differences in the state of the muscle. Even at a high concentration the rate of depolarization may vary from one experiment to the other. Thus, *e. g.*, in three experiments using a concentration of 1.0 M the membrane was depolarized after 15 min of ethanol exposure to levels that were 12, 15 and 18 per cent, respectively, below the initial membrane potential values.

The other method used for the studies of membrane potential changes implied measurements from different muscle fibres in rapid succession during the whole course of ethanol action. This experimental procedure was chosen in order to provide further evidence that the depolarization is not due to a combined effect of ethanol and the injury gradually inflicted to the cell

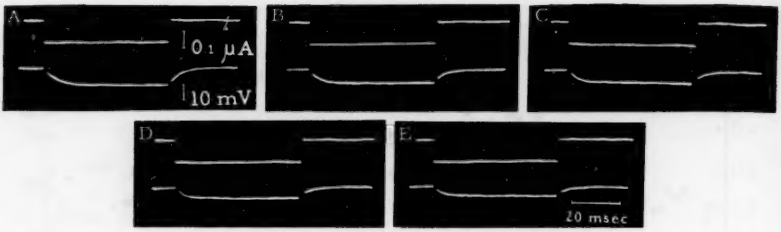

Fig. 5. Ethanol effects on voltage-current relation (V: I) as determined by square pulse analysis. *A*, immediately before, *B*—*E* 5, 10, 15 and 20 minutes, respectively, after administration of ethanol (concentration 1.0 M). Inward current pulses on upper trace, changes of membrane potential (hyperpolarization) on lower trace of each pair. Calibrations in *A* and time bar in *E* apply to all records. Internal electrodes 0.4 mm apart. Membrane potential at start of experiment 88 mV, after ethanol exposure for 20 minutes 66 mV.

during prolonged microelectrode recording. An experiment of this kind is illustrated in Fig. 4. Measurements of nine fibres show a range of the recording values before ethanol administration between 70 and 85 mV, most of the values being about 80 mV (*A*). After adding ethanol to the bath up to a concentration of 1.2 M (*B*), the membrane potentials were successively reduced to values around 40 mV after about 15 min. Eighteen minutes after ethanol administration the drug was washed out, the muscle soaked in Ringer's solution, and then other cells were tested (*C*). A gradual rise of the values could then be observed. About 50 min after the drug had been washed out, the values of the recordings were between 60 and 80 mV, the highest values thus observed being comparable to the membrane potential values before ethanol administration.

In a similar experiment, using a lower concentration, 0.1 M, a depolarization occurred from 72—80 mV to 50—63 mV during exposure to ethanol for 30 min. At a high concentration, 1.7 M, the depolarization seemed to be of almost the same order of magnitude as at 1.2 M, whereas the repolarization of the membranes after removal of the ethanol was significantly slower.

II. Membrane resistance changes

In order to study the changes in membrane resistance caused by ethanol, the voltage-current relation has been determined by inserting two microelectrodes into the same cell, one to pass current, the other to record the voltage changes induced (cf. Methods). The electrodes have been kept in the same position throughout the experiment, an arrangement which makes it possible to study relative changes of the effective resistance of the muscle fibre but does not give the data necessary to calculate the numeric value of the transverse resistance of the membrane; for this purpose measurements have to be made at varying electrode distances. As, however, certain errors are introduced into the measurements when a muscle fibre exposed to ethanol is subjected to repeated penetrations, determinations of the numeric value of the transverse resistance

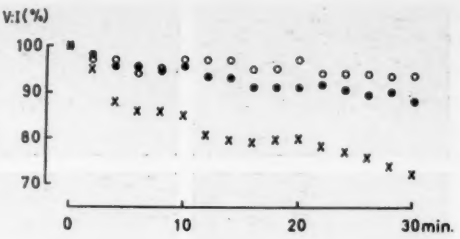

Fig. 6. Diagrammatic representation of ethanol effects on voltage-current relation (V: I), determined as in Fig. 5. V:I ratio in per cent of mean value before administration of ethanol [concentrations 0.1 M (o), 0.2 M (•) and 0.5 M (x)]. Internal electrodes less than 0.1 mm apart in all three experiments. Time in minutes from moment of ethanol administration.

have not been included in the present investigation. To what extent the changes observed in voltage-current relation may be referred to changes in the transverse resistance will, however, be discussed below.

Fig. 5 shows records from an experiment using hyperpolarizing square pulses of 0.1 μA and a duration of 50 msec. *A*, recorded before ethanol administration, shows a membrane potential change of 10 mV after the stable level of the electrotonic potential had been reached. The relation between this potential change and the current applied is 10^5 (Ω). *B*—*E* are records made 5, 10, 15 and 20 min, respectively, after adding ethanol to the muscle bath. The stable potential changes were 8, 7, 6 and 5 mV, respectively, revealing a gradual decrease of the V: I ratio to 50 per cent of its initial value after ethanol exposure for 20 min.

In Fig. 6 the results from three typical experiments of this kind have been diagrammatically represented. The ethanol content in the different experiments was 0.1, 0.2 and 0.5 M respectively. One record was made about every 2 min and the relation V: I was estimated. During exposure to ethanol in the concentration 0.5 M the V: I raio gradually decreased and after 30 min amounted to 72 per cent of the initial value. At a concentration of 0.2 M there was a slower decline of the V: I ratio, *viz.* by about 12 per cent in 30 min, whereas in experiments using 0.1 M the decline during that time was 6 per cent which corresponds to the range of variations observed in measurements on muscle in Ringer's solution for the same period of time. A greater change in the V : I ratio at higher ethanol content was typical of all experiments (cf. Fig. 5) and was most clearly seen when the ethanol concentration was increased stepwise in one and the same experiment.

The second way of measuring changes in the V: I ratio, using gradually increasing current (see Methods), corresponds to the determinations by means of a whole series of positive and negative square pulse stimulations of different amplitudes provided that the continuous increase of the current is sufficiently slow. The time 1 sec for increase from — 0.2 μA to 0.2 μA proved to be slow enough, giving the same V: I relation as by application of a series of current pulses. Fig. 7 shows an experiment of this type in which the current has been gradually changed from about — 0.2 μA to about 0.2 μA, resulting in a concomitant change from 20 mV hyperpolarization to 20 mV depolarization. The

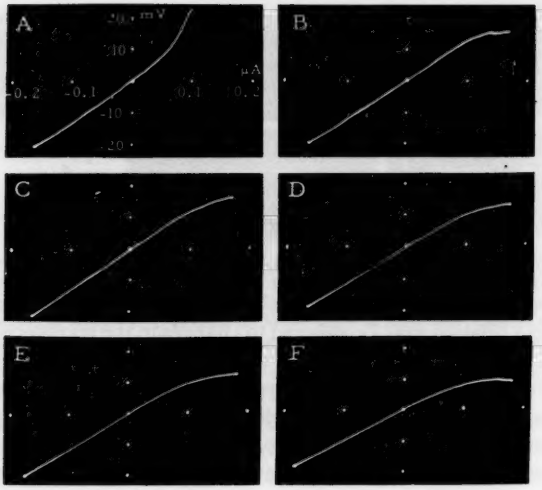

Fig. 7. Direct recording of voltage-current curves obtained before (*A*) and after exposure to ethanol for 5 (*B*), 10 (*C*), 15 (*D*), 20 (*E*) and 25 (*F*) minutes. Oscilloscope spot tracing change in membrane potential (vertical deflexion) as a function of polarizing current (horizontal deflexion). Gradual increase of polarizing current in the course of about 1 sec. Inward currents and hyperpolarization of membrane in lower left-hand quadrants, outward currents and depolarization in upper right-hand quadrants. Ethanol concentration 0.5 M. Internal electrodes 0.06 mm apart. Calibrations in *A* apply to all records.

slope of each curve is constant, except when the membrane is depolarized more than 10 mV. After ethanol administration there is a decrease in the linear part of the slope, implying a reduction of the ratio V: I in agreement with the results obtained by square pulse analysis. There also occurs a progressive flattening of the voltage-current curve when outward currents of high intensity (> 0.1 μA) are passed. The cause of this phenomenon, which seems to be connected with activation processes (cf. JENERICK 1959), will not be dealt with in this work.

The changes in voltage-current relation imply that the effective resistance of the fibre has been reduced. Most likely this reduction can be referred to a change in transverse membrane resistance. However, since it is well known that ethanol passes rapidly into cells (OVERTON 1902) an ethanol effect on the internal resistance has also to be considered. An attempt has been made to estimate this effect as per following, on the basis of the assumption that the ethanol effects on the resistivity of Ringer's solution are comparable to those on the internal resistivity of the muscle cell, in spite of the considerably lower value of the latter (BOZLER and COLE 1935).

A series of determinations, by an ac bridge method, of the resistivity of Ringer's solution showed that ethanol contents of 0.5 M and 1.0 M raised the resistivity values by 10 and 16 per cent respectively. Paying no regard to ionic distribution changes secondary to increased permeability, the influence of such an increase of the internal resistivity on the V : I ratio can be estimated by means of the equation 2 and the membrane constants given in the paper by FATT and KATZ (1951). The calculations showed that an increase of the internal

resistance by 10 or 16 per cent would result in an increase of the effective resistance of the fibre by 5 and 8 per cent respectively. However, the experimental data actually obtained show a decrease of the ratio V: I by about 30 and 50 per cent at the corresponding ethanol content in the muscle baths. It seems therefore, on the basis of the data available at present, that the ethanol effects are mainly to be ascribed to a change in transverse resistance of the muscle fibre membrane.

Discussion

In a brief report on the influence of various toxic substances on the membrane potential of frog muscle fibres measured by means of intracellular technique, TAUC (1952) stated that the effect of 1 M ethanol on the membrane potential was quite insignificant, no depolarization being recorded during the first 15 min and a change of about 10 per cent being observed after 90 min. It has not been possible to compare TAUC's results with those obtained in the present investigation, as the experimental data given in his report are too scarce. It can only be suggested as a possibility that the difference in results is due to differences in the oxygenation of the muscle baths. In the present series of experiments it has been found that ethanol-induced depolarization takes place very slowly under anoxic conditions and is then of the same order of magnitude as reported by TAUC.

The results obtained in earlier studies of demarcation potentials in frog nerve exposed to ethanol (GALLEGO 1948, POSTERNAK and MANGOLD 1949) correspond in some respects with those found in the present investigation using direct measurements of membrane potential changes in muscle. Thus, recordable changes appear at around the same threshold dose of about 0.1 M, and both in nerve and muscle there is an increase in depolarization with increasing ethanol content. In view of the different recording techniques used, it is however not possible to make quantitative comparisons of the membrane potential changes observed in the two types of tissues.

There is a close correspondence between the ethanol concentration necessary to produce the changes demonstrated in membrane potential and the concentration which, as has been shown by BLUME (1925), results in a lowering of the threshold on direct stimulation of frog muscle. To the threshold rise appearing at higher ethanol content correspond an increased depolarization and a further reduction of the membrane resistance. In analogy with GALLEGO's results on nerve quoted above, both the initial increase and the subsequent reduction of the muscle excitability may be explained by a reduction of the membrane potential.

According to generally accepted views (cf. USSING 1960), the potential difference between the inside and the outside of the muscle fibre is related to the concentration gradients of the diffusable ions and their permeability coefficients. The increase in membrane conductance during ethanol action, implying an

increase of the membrane permeability to one or more ions, may thus be presumed to be a primary cause of the changes of the membrane potential during ethanol exposure. The fact that an increase in the permeability to potassium or chloride ions will tend to increase the membrane potential, whereas an increase in the permeability to sodium ions will cause a depolarization of the membrane, seems to suggest that the membrane permeability to sodium ions is affected during ethanol exposure. However, before conclusive evidence can be obtained whether ethanol affects the permeability to any particular ion, it is obviously necessary to undertake experiments especially designed to evaluate the effects of ethanol on the membrane permeability to different ions.

The author is greatly indebted to Dr C. R. SKOGLUND for continual advice and encouragement and to Mr L. HAAPANEN for construction of apparatus.

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Stratification of Absorbed Solutions in Mesenterial Veins[1]

By

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Abstract

ÖSTLING, G. *Stratification of absorbed solutions in mesenterial veins.* Acta physiol. scand. 1961. *52.* 254—266. — Stratification in the mesenterial venous blood of solutions which have been absorbed from the small intestine was demonstrated. Isotonic and hypotonic sodium phosphate solution and water were transported in the upper, and hypertonic glucose and saline solutions in the lower streamlines in the mesenterial veins. These observations show that the blood stream is laminar and that the streamline flow demonstrated previously in the portal vein after injection of dyes etc. also occurs in the mesenterial part of the venous portal circulation after intestinal absorption. No stratification could be demonstrated in the mesenterial veins after absorption of isotonic glucose and sodium chloride solution. The reasons for the presence and absence of stratification are discussed and different absorption mechanisms are considered. It is suggested that stratification depends on "intestinal short-cut absorption". According to this hypothesis, under certain conditions translocation from the intestinal lumen of intraintestinally instilled solutions to the capillaries takes place via gaps between the mucosal epithelial cells. These gaps are produced by expulsion of cells from the epithelial border during the absorption.

Stratification in the peripheral circulation of intravascularly administered solutions and suspensions occurs under certain conditions (ÖSTLING 1952). As these observations have been published only in Swedish the phenomena will be described briefly here. Examples are given of flotation, sedimentation and absence of stratification of solutions in the mesenterial vein after their absorption from the small intestine. An attempt is made to explain these phenomena.

[1] A preliminary report of this work was presented at the IX Scandinavian Physiological Congress 1957.

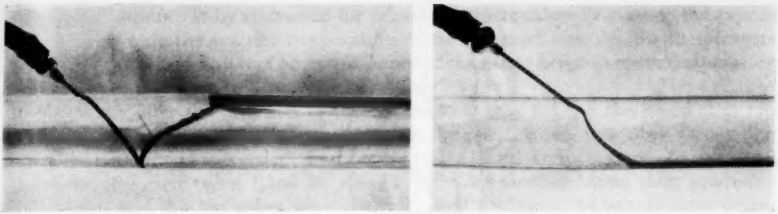

Fig. 1 (left). *Flotation:* when Indian ink diluted with water is injected slowly into a plastic tube with flowing 20 per cent saline solution, the dye rises and is carried in the topmost streamlines.

Fig. 1 (right). *Sedimentation:* when Indian ink diluted with 20 per cent saline solution is injected slowly into a plastic tube with flowing water, the dye settles and moves in the lowest streamlines.

The stratification phenomenon

Fig. 1 illustrates the flotation and sedimentation phenomena. Flotation originates when a solution or suspension flows slowly into a laminar (streamline) stream of fluid of higher specific gravity. If, on the other hand, the fluid flowing in slowly has a higher specific gravity than the fluid in the plastic tube, sedimentation occurs. If the stream in the tube is not laminar but turbulent, no stratification occurs. It can be seen that the Indian ink introduced is distributed in eddies through all the strata in the plastic tube.

Stratification can also be demonstrated intravascularly in both test animals and man (ÖSTLING 1952). If suspensions of Indian ink are injected slowly into a well-exposed and illuminated jugular vein in rabbit, flotation in the blood stream can be seen when the Indian ink is diluted with water and sedimentation when the Indian ink is diluted with 20 per cent saline solution. When thin blood vessels are used for stratification experiments the blood is allowed to run from the vessel some distance from the injection site into a test tube via a needle. When water-diluted Indian ink is used, the topmost streamlines of the blood which run from the needle are black and the black color accumulates in the topmost strata of the blood in the collection tube. When Indian ink of higher specific gravity than blood is used the black color is found in the lowest streamlines and the Indian ink runs down and accumulates on the bottom of the tube. If radioactive phosphate is used instead of Indian ink in the experiment the greatest part of the radioactivity accumulates in the topmost or the lowest strata, depending on the specific gravity of the solutions. Using labelled phosphate solutions, stratification has been established following slow injections into peripheral veins and arteries, and also in the femoral and renal veins after injection into the femoral and renal arteries respectively (ÖSTLING 1952). These observations concur with the generally held view that the blood stream in arteries and veins is mostly laminar (BURTON 1952) and seem to indicate that streamline flow also occurs in capillaries.

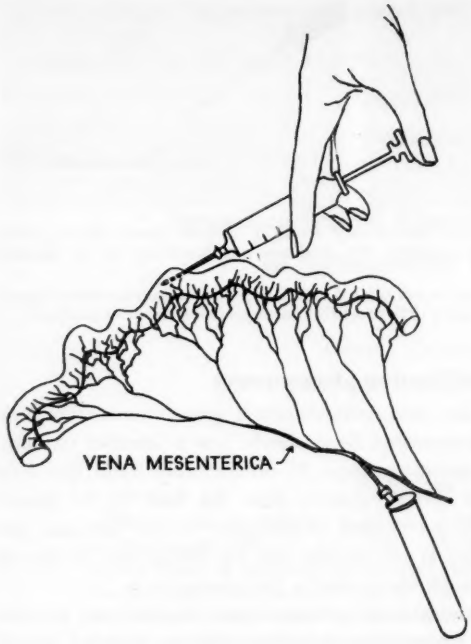

Fig. 2. *Sketch illustrating the absorption experiments.* A needle is introduced into the mesenterial vein at a point where it drains a 10—15 cm long portion of the small intestine. 5 ml of the test solution is then instilled intraluminally into the middle of this section of the intestine and the blood from the vein is collected into test tubes.

Stratification occurs in the blood stream after slow intravascular instillation of solutions. During absorption from intestine there is also a slow translocation into the blood vessels. The question of whether solutions stratify after absorption from the intestine has not been studied.

Methods

Heparinized rabbits weighing 2—2.5 kg were used in the absorption experiments. The animals were anesthetized with an intravenous injection of 8—10 ml of 25 per cent urethane solution or by ether inhalation. The anesthetic used did not affect the experiments. As the urethane injections produced hemolysis, ether anesthesia was employed when examining absorption of pure water.

The abdomen was opened and a suitable intestinal loop exposed. The part of the ileum lying about 20 cm orally from the cecum was used most frequently. In a few experiments the jejunum about 20 cm from the stomach was employed. This part of the intestine was more difficult to use because the mesenterial veins are thinner and more fragile here than in the ileal tract. An injection needle of the same diameter as the vessel was inserted in the mesenterial vein at a point where it drained a 10—15 cm long section of the small intestine (Fig. 2). Immediately afterwards 5 ml of the test solution was rapidly injected into the middle of the selected portion of the intestine. The intestine was handled as little as possible throughout the experiment. It was neither rinsed nor emptied in any way. The experiments were not affected by the degree of

filling of the intestine or by starvation for periods of different length prior to the experiment. Neither were the exterior temperature of the intestinal loop nor the temperature of the solution injected into it of any significance in the range between room temperature and 38° C.

Immediately after the injection of the test solution into the gut the blood that ran from the needle in the mesenterial vein was collected into the 5 ml test tubes. Depending on how the experiment succeeded, 4—12 tubes full of blood were obtained. In successful experiments the first tubes filled in 10—15 sec. The flow of blood then gradually slowed down and the last tubes were changed after 1—3 min. In experiments where the flow was sluggish the first tubes filled in 1—2 min and last in 5—10 min. It was important that the needle had the same internal diameter as the vein, and that the blood from the needle ran down in an unbroken stream along the inner side of the test tube. After filling, the test tubes were placed in a rack, care being taken not to let shaking disturb possible stratification. Immediately the blood sampling was completed, the uppermost fourth of the sample was withdrawn with a pipette. The following fourths were then drawn off in turn. The contents of the test tubes were thus divided into four roughly equal strata. Each separated stratum thus obtained was then mixed by shaking vigorously.

The following solutions were employed in the absorption experiments:
Hypotonic sodium phosphate solution (0.15 g $Na_2HPO_4 \cdot 2H_2O$/100 ml H_2O)
Isotonic sodium phosphate solution (1.5 per cent)
Isotonic glucose solution (5 per cent) without and with admixture of phosphate
Hypertonic glucose solution (50 per cent) without and with admixture of phosphate
Isotonic sodium chloride solution (0.9 per cent) with admixture of phosphate
Hypertonic sodium chloride solution (20 per cent) with admixture of phosphate
Distilled water and tap water with and without admixture of carrier-free P^{32}.

The hypotonic and isotonic sodium phosphate solutions contained 300—700 μC of radioactive phosphate. The glucose and saline solutions were labelled by adding 0.1—0.5 ml (300—700 μC) of isotonic sodium phosphate solution. The osmolarity of the hypertonic solutions thus varied slightly from one experiment to another, but was always hypertonic in relation to the blood plasma. In some of the experiments using water 300—700 μC of P^{32} was added in the form of a couple of drops of carrier-free P^{32} solution (obtained from Amersham, England).

The radioactivity in the blood samples was measured with the aid of a mica window Geiger-Müller tube. For this purpose 0.02 ml aliquots of whole blood were pipetted onto round discs of filter paper which were glued to the bottom of aluminum planchets. In the experiments with glucose, the glucose content of the layers was determined by the method of HAGEDORN and JENSEN (1954). In the experiments with distilled water and tap water, the blood samples were centrifuged at 2,000 rpm. Whether or not the plasma had stained red by hemolysis was assessed by mere inspection.

The stratification of the solutions used in this paper was controlled after slow injection direct into rabbit veins. Flotation in venous blood was observed with distilled and tap water, hypotonic sodium phosphate solution and isotonic sodium phosphate, glucose and sodium chloride solutions; sedimentation was established with hypertonic glucose and sodium chloride solutions.

Results

Flotation

Ten rabbits were used in the experiments. Table I shows the stratification in the blood of the mesenterial vein in one of the experiments in which isotonic sodium phosphate solution was injected into the intestinal lumen. The radio-

Table I. P^{32}-flotation in mesenterial venous blood after injection of isotonic sodium phosphate solution into the small intestine

	Number of test tube									
	1	2	3	4	5	6	7	8	9	10
Stratum I (topmost), cpm/0.02 ml	87	124	225	415	902	1,893	3,532	4,626	6,542	6,606
Stratum II, cpm/ 0.02 ml.....	44	81	83	197	299	897	1,291	2,130	2,858	4,260
Stratum III, cpm/ 0.02 ml.....	21	19	21	52	100	235	369	792	835	1,272
Stratum IV, cpm/ 0.02 ml.....	8	6	7	32	59	164	336	540	594	678
Sampling time in sec.	20	25	30	35	45	50	60	65	80	80

Table II. P^{32}-flotation in mesenterial venous blood after injection of hypotonic sodium phosphate solution into the small intestine

	Number of test tube									
	1	2	3	4	5	6	7	8	9	10
Stratum I, cpm/ 0.02 ml.....	81	332	845	828	862	1,430	2,068	2,446	2,917	3,707
Stratum II, cpm/ 0.02 ml.....	53	193	400	544	591	628	649	970	1,523	1,914
Stratum III, cpm/ 0.02 ml.....	24	102	210	287	434	359	396	493	615	481
Stratum IV, cpm/ 0.02 ml.....	7	96	151	186	206	244	320	204	343	228
Sampling time in sec.	10	20	20	25	25	35	35	50	70	85

activity is obviously greatest in the topmost stratum of each tube, decreasing towards the bottom. Flotation was observed in all the experiments. In some the flotation was slightly more and in others slightly less pronounced than in the case described in Table I.

Eight experiments were made with hypotonic sodium phosphate solution. Flotation was as pronounced as with isotonic phosphate solution. Table II shows the results of one of the experiments.

Twenty-nine experiments were made with distilled water or tap water. Table III gives the results of an experiment with distilled water mixed with carrier-free P^{32} solution. Flotation of radioactivity and water was observed in this experiment. The red color of the plasma in the uppermost layers derived

Table III. Flotation in mesenterial venous blood after injection of distilled water containing carrier-free P^{32} into the small intestine

	Number of test tube						
	1	2	3	4	5	6	7
Stratum I, cpm/0.02 ml..	[1]+ 300	++ 3,354	++ 4,047	++ 5,304	++ 9,894	+++ 6,045	++++ 12,423
Stratum II, cpm/0.02 ml..	+ 142	+ 1,769	+ 3,042	++ 3,564	++ 4,260	++ 2,646	++ 2,700
Stratum III, cpm/0.02 ml..	29	1,131	2,013	3,039	+ 2,565	+ 1,695	++ 1,065
Stratum IV, cpm/0.02 ml..	31	864	1,563	2,316	2,250	+ 1,230	+ 1,377
Sampling time in seconds	20	30	35	40	45	55	60

[1] The + symbols indicate the intensity of red-staining of the plasma.

from hemoglobin liberated by hemolysis. Flotation — indicated by visible red-staining of plasma — was not as regular a phenomenon as in the experiments with isotonic or hypotonic sodium phosphate solution. In 3 exp. red-staining was not observed in a single one of the blood samples taken. In 15 exp. red flotation was observed in all the tubes and in the remaining 11 tests the topmost layer of plasma stained red in 1—4 of the blood samples. In 3 of these 11 exp. the water was labelled with carrier-free P^{32} solution and typical P^{32}-flotation was established in each test tube although flotation of color was seen only in 1—1—4 tubes, respectively. These findings indicate that the water had risen to the upper strata but the hemolysis was so weak that it was impossible to detect the red color with the naked eye.

Conclusion: Isotonic and hypotonic sodium phosphate solutions and water are carried in the upper streamlines of the blood in the mesenterial veins after absorption from the small intestine.

Sedimentation

Fifteen rabbits were given hypertonic glucose solution containing P^{32}. Sedimentation was observed in every tube in all the experiments. Table IV gives the result for an experiment in which the glucose content and radioactivity of the layers were determined. Both glucose and radioactivity showed sedimentation. No quantitative correlation was observed between glucose and radioactivity sedimentation in this or the other experiments. In some experiments, in which 50 per cent glucose solution without P^{32} was used, the glucose sedimentation was of the same magnitude as in Table IV.

Table IV. Sedimentation in mesenterial venous blood after injection of hypertonic glucose solution containing P^{32} into the small intestine

	Number of test tube				
	1	2	3	4	5
Stratum I, Glucose content: mg/100 ml..	158	146	146	162	174
Stratum II, Glucose content: mg/100 ml..	158	178	146	178	226
Stratum III, Glucose content: mg/100 ml..	172	178	206	232	252
Stratum IV, Glucose content: mg/100 ml..	190	194	208	240	268
Stratum I, P^{32} content: cpm/0.02 ml....	1	4	9	20	40
Stratum II, P^{32} content: cpm/0.02 ml....	8	20	40	81	131
Stratum III, P^{32} content: cpm/0.02 ml....	30	43	90	160	266
Stratum IV, P^{32} content: cpm/0.02 ml....	69	94	133	228	463
Sampling time in seconds	40	45	60	95	130

Table V. P^{32}-sedimentation in mesenterial venous blood after injection of hypertonic sodium chloride solution containing P^{32} into the small intestine

	Number of test tube						
	1	2	3	4	5	6	7
Stratum I, cpm/0.02 ml..	43	54	62	38	71	100	187
Stratum II, cpm/0.02 ml..	32	68	98	99	180	236	388
Stratum III, cpm/0.02 ml..	45	83	157	264	425	429	657
Stratum IV, cpm/0.02 ml..	58	129	265	613	681	727	1,031
Sampling time in seconds	30	45	65	85	100	120	160

Table V is from an experiment with hypertonic saline solution with P^{32} addition. The radioactivity was highest at the bottom of the columns of blood. The series comprised 7 exp. Sedimentation was observed in all of them. In 2, however, there were individual tubes in which the radioactivity was almost evenly distributed throughout the column of blood.

The radioactivity only and not the NaCl content was determined in these experiments with hypertonic saline solution containing P^{32}. The results obtained from the experiments with glucose solution and water containing P^{32} lend support to the assumption that the sedimentation demonstrated did not comprise solely the labelled phosphate but the solution as a whole.

Conclusion: Hypertonic glucose and saline solution are transported in the lower streamlines of the blood in the mesenterial veins after absorption from the small intestine.

Table VI. No P^{32}-stratification in mesenterial venous blood after injection of isotonic glucose solution containing P^{32} into the small intestine

	Number of test tube							
	1	2	3	4	5	6	7	8
Stratum I, cpm/0.02 ml..	215	240	454	429	634	675	662	571
Stratum II, cpm/0.02 ml..	278	458	444	534	587	633	693	695
Stratum III, cpm/0.02 ml..	254	381	496	557	563	645	818	742
Stratum IV, cpm/0.02 ml..	183	296	371	517	645	620	754	762
Sampling time in seconds	25	30	30	35	40	50	55	65

Table VII. No P^{32}-stratification in mesenterial venous blood after injection of isotonic sodium chloride solution containing P^{32} into the small intestine

	Number of test tube						
	1	2	3	4	5	6	7
Stratum I, cpm/0.02 ml..	618	473	386	398	428	390	458
Stratum II, cpm/0.02 ml..	516	509	419	434	420	398	435
Stratum III, cpm/0.02 ml..	435	556	579	518	454	436	403
Stratum IV, cpm/0.02 ml..	316	744	668	542	465	389	320
Sampling time in seconds	25	30	35	30	40	45	50

Absence of stratification

Isotonic glucose solution with P^{32} added was used in 11 exp. Table VI, which gives the results for one of them, gives no evidence of the occurrence of stratification. Nine of the experiments gave similar results irrespectively of whether radioactivity or glucose content was determined. Sedimentation appeared to occur in 2 of the tests. It was not so pronounced, however, as when hypertonic glucose and saline solution was administered. As mentioned previously, isotonic glucose solution rises to the upper streamlines after direct slow injection into the peripheral veins.

Table VII shows the result of an experiment with isotonic sodium chloride solution containing P^{32}. No stratification of radioactivity could be established. Of the 12 exp. performed, 11 gave the same result. One experiment showed an equally pronounced flotation as with isotonic or hypotonic phosphate solution. The result was obtained too late to permit an examination of the injection solution used to eliminate the possibility that the solution had been wrongly prepared.

Conclusion: Isotonic glucose and sodium chloride solutions showed no stratification and were evenly distributed in the mesenterial venous blood stream after absorption from the small intestine.

Discussion

The present investigation shows that isotonic and hypotonic sodium phosphate solutions of lower specific gravity than blood and water are transported in the upper streamlines of the mesenterial venous blood stream after absorption from the intestine. Hypertonic glucose and saline solutions of higher specific gravity than blood are carried in the lower streamlines after translocation from the intestinal lumen. The laminar (streamline) blood flow in the portal vein, which prevents blood from the different internal organs from mixing prior to arrival in the liver (COPHER and DICK 1928, HAHN *et al.* 1945), thus also occurs in the mesenterial veins during absorption.

The intravascular hemolysis observed by LEE (1954) after absorption of water is verified by the present observation of water flotation, which causes hemolysis in the topmost streamlines in the mesenterial veins. Flotation of water in blood *in vitro* has previously been demonstrated (ÖSTLING and NYBERG 1952).

After translocation from the intestine to the veins certain solutions are carried further in the upper or lower streamlines of the mesenterial blood. Thus, the solutions are distributed in the blood stream just as if they had entered the circulation in a slow flow from the intestinal lumen via narrow openings (pores). The fluid circuit theory of INGRAHAM and VISSCHER (1938) assumes that there is a flow of fluid through pores of different sizes between the intestinal lumen and the capillaries and vice versa. The idea of the existence of pores in the intestinal mucosa has been revived recently (CURRAN and SOLOMON 1957, PARSONS and WINGATE 1958, DURBIN *et al.* 1958). CURRAN and SOLOMON (1957) write as follows: "The epithelial membrane may be described in physical terms as equivalent to a membrane with uniform negatively charged right circular pores of 36 Å radius, occupying 0.001 per cent of the surface area." This size of the radius is of interest because PAPPENHEIMER (1953) calculated that for membranes with a greater effective radius of pore than c. 20 Å the net flow of water through diffusion is negligible compared with hydrodynamic flow caused by hydrostatic or osmotic pressure. DURBIN *et al.* (1958) suggest that the pores in the ileum are intercellular. Earlier physiological investigations thus indicate that there may be pores in the intestinal mucosa through which the intestinal contents flow into the capillaries.

The ultrastructure of the intestinal mucosa and intestinal capillaries has been studied in the last few years by electron microscopy (ZETTERQVIST 1956, BENNETT *et al.* 1959, PALAY and KARLIN 1959 a). No pores forming a direct connection between the intestinal lumen and the capillaries have been established. Fenestrations or pores of a radius of 100—250 Å occur in the endothelial cells of the capillaries. Counting from the lumen, between the intestinal lumen and these pores there are: "(1) the apical surface and striated

border of the epithelium, (2) the cytoplasm of the epithelium, (3) the basal or lateral surfaces of the epithelial cells, (4) the basement membrane underlying the epithelium, (5) the loose connective tissue of the lamina propria, (6) the basement membrane surrounding the capillaries" (PALAY and KARLIN 1959 a). For flow of intestinal fluid from the lumen into the capillaries to be possible it is necessary to postulate that these tissues can be passaged in some way so that the unchanged intestinal contents enter the pores of the capillary endothelium.

A possible way in which unchanged intestinal contents can pass via the epithelial layer is pinocytosis (PALAY and KARLIN 1959 b, CLARK 1959, CRANE 1960). As there is no confirmation that pinocytosis plays a role in the absorption of solutions employed in the present study, this transport mechanism must be rejected for the present.

Another way in which the intestinal contents might circumvent the epithelial cells can be deduced from RITTER's (1957) investigation of expulsion of epithelial cells from intestinal mucosa. Microscopy of living intestinal mucosa at 50—100 × magnifications showed that epithelial cells were expelled when different nutritive solutions were dropped on the mucosa. When 1—2 cells were freed small gaps were produced and were closed almost instantaneously by filling with adjacent cells. In the event of major defects in the epithelium (4—5 cells) it took $^1/_2$—1 hour before the openings were covered. According to RITTER, a continuous expulsion and regeneration of epithelial cells occurs in the villi during absorption. Observations by the isotope technique that the epithelial cells of the intestine regenerate in 24—48 hours concur with this finding (LEBLOND *et al.* 1957, HUGHES *et al.* 1958).

The possibility that absorption can occur via a short-cut through gaps left by expelled epithelial cells is supported by some observations. JASSINOWSKY (1925) found numerous epithelial cells in the Ringer's solution used to rinse the small intestine of rabbit. He estimated that 5,000—7,000 cells were freed per minute and sq. cm. Expulsion of epithelial cells thus occurs in rabbits, the species employed in the present investigation. RITTER (1959) observed that the extent of expulsion varied with the substances administered. Hypertonic solutions such as 50 per cent glucose and 10 per cent saline cause considerably more active expulsion of cells than the same solutions in isotonic concentrations. With hypertonic solutions the defects in the epithelial layer in villi increase in number and size and repair more slowly. This gives the intestinal contents greater chances of coming into contact with the subepithelial tissues. Hypertonic glucose and saline solutions showed pronounced stratification in the present experiments after absorption, whereas no stratification could be demonstrated when the corresponding isotonic solutions were used. One may therefore assume a correlation between vigorous cellular expulsion and stratification, on the one hand, and limited cellular expulsion and no stratification on the other hand. It can be inferred from this

that solutions which provoke pronounced expulsion of cells can pass the epithelial border of the intestinal wall and flow via the openings produced.

But there is still the translocation through the basement membranes and the interjacent loose connective tissue before the pores of the capillaries are reached. The basement membranes, judging by electron microscopy, seem to be almost homogenous but of low opacity and to have neither pores nor meshwork. It has been pointed out recently, however, that the basement membranes are composed of a condensation of fine fibrilla (PALAY and KARLIN 1959 a). It seems that the basement membranes have a very subtle structure which is either difficult to demonstrate by existing techniques or which is destroyed postmortally. The possibility that fluid may flow through the basement membranes by an unknown mechanism (electro-osmosis?) cannot be disregarded entirely, especially as it has not been possible to demonstrate any opaque structural barrier. The hypothetical short-cut absorption via gaps in the epithelial border of the villi produces flotation or sedimentation depending on whether the specific gravity of the intestinal contents is lower or higher than that of the capillary blood. Solutions which are carried evenly dispersed in the venous stream following the absorption pass, according to this hypothesis, all the layers of mucosa including the epithelial cells which obstruct direct flow from the intestinal lumen to the capillaries. Short-cut absorption would thus seem to involve stratification, whereas translocation through the epithelial cells inhibits it.

Stratification can theoretically originate also in each junction of the mesenterial veins and of the veins and the capillaries when two blood streams of different specific gravity combine. The stem of the vein from which the blood samples were taken drained a 10—15 cm long piece of the intestine. The test solutions were always injected into the middle of the intestinal sector (see fig. 3). No peristalsis was observed in at least 90 per cent of the experiments. The 5 ml injected could therefore not be distributed evenly throughout the lumen, and the concentration was greatest around the injection site. The veins of this part, consequently, were able to absorb more of the instilled solution than the veins of the outer parts of the intestinal loop employed. Hence, although the conditions for stratification should always have been present it could not be demonstrated with either isotonic glucose or saline solution. The possibility that stratification originates in the greater branches of the venous tree, close to the stem used for the sampling, must therefore be precluded. For the same reasons, the origin of stratification at all other levels in the mesenterial veins and capillaries can also be eliminated.

The stratification of solutions in mesenterial venous blood following absorption from the intestine is difficult to account for by current absorption theories. Short-cut absorption, *i. e.* passage of the intestinal contents via gaps left by expelled cells in the marginal epithelial layer of the mucosa, offers a hypothetic explanation of the phenomenon. Stratification has been demonstrated in the large mesenterial veins, but it probably occurs in the entire mesenterial venous system. If this is true, it must be assumed that there is a

flow of the intestinal fluid into the capillaries via the pores in the capillary endothelium. The powers that drive the stream cannot be envisaged. Hydrostatic pressure can probably be ruled out as peristalsis was seen very seldom during the present absorption experiments and as stratification was usually more pronounced the later the blood samples were taken (see the tables).

Absorption via the intercellular area between adjacent epithelial cells has been discussed previously. Nothing has been observed to confirm such a route of absorption (ZETTERQVIST 1956). Short-cut absorption via gaps in the epithelial coating of the villi has not been proposed earlier to the present author's knowledge. RITTER (1957), who saw gaps in the living mucosa, was unable to establish any correspondence in the histological preparations obtained postmortally, although he examined hundreds of sections. In the classical histological picture the villi are covered by an unbroken row of epithelial cells. This does not, however, preclude the appearance of openings *in vivo* in the epithelial border during absorption. The villi are made up of soft, mobile tissue which performs pumping movements. It is therefore possible that the villi collapse after death, pressing the epithelial cells together and effacing the gaps.

These attempts to account for the origin of the stratification phenomenon are based on weak evidence. The hypothesis of intestinal short-cut absorption must therefore be regarded as merely a working hypothesis.

I am greatly indebted to miss KERSTIN FROSTELL for technical assistance and docent RALPH GRÄSBECK for criticism of the manuscript.

Addendum

C. P. LEBLOND (Classical technics for the study of the kinetics of cellular proliferation in *The kinetics of cellular proliferation*, edited by F. STOHLMAN. GRUNE & STRATTON. New York and London 1959. p. 35—37. See also Anat. Rec. 1958. *132.* p. 247.) has observed in man, cat, rabbit and mouse at the tips of the villi *extrusion zones* with numerous irregularities, interrupted striated border and shriveled cells pushed above the regular epithelial lining. The occurrence of such extrusion zones seems to support the intestinal short cut absorption hypothesis.

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Human Serum Cholinesterase as a Sialo-Protein

By

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Abstract

SVENSMARK, O. *Human serum cholinesterase as a sialo-protein.* Acta physiol. scand. 1961. *52.* 267—275. — Incubation of human serum with sialidase (neuraminidase, receptor destroying enzyme) results in a marked decrease of the mobility of cholinesterase without affecting the enzymatic activity as indicated by the splitting of acetylcholine and butyrylcholine. The results suggest that human serum cholinesterase is an acid glycoprotein containing several sialic acid residues per molecule. In normal serum sialidase could not be detected whereas spinal fluid showed weak sialidase activity. In a single pool of serum from patients a marked sialidase activity was observed.

Knowledge of the chemical properties of human serum cholinesterase (pseudocholinesterase, unspecific cholinesterase) is scarce as this enzyme has not yet been isolated in a pure state. In a purified preparation considerable amounts of carbohydrate were found but the impurities still present made it impossible to identify the cholinesterase as a glycoprotein (SURGENOR and ELLIS 1954). Even from impure preparations information concerning the properties of cholinesterase can be obtained when its enzymatic activity is used to trace the protein. Thus, the electrophoretic mobility determined on paper at pH 9 was between that of α_2- and of β-globulins (GREGOIRE and DERRIEN 1952). In chromatography of serum on diethylaminoethyl-cellulose (DEAE-cellulose) cholinesterase appeared between albumin and the acid glycoprotein coeruloplasmin (SVENSMARK 1961) suggesting that the enzyme is an acid protein.

The acidity of acid α_1-glycoprotein is due to sialic acid residues which can be split off the protein by bacterial sialidase (neuraminidase) whereby the mobility of the protein is considerably reduced (POPENOE and DREW 1957).

I have investigated whether the acidity of cholinesterase is due to sialic acid by using the effect of sialidase on the electrophoretic mobility of the protein as a criterion for the presence of sialic acid residues. This was feasible because the enzymatic activity of cholinesterase was not abolished by the treatment with sialidase.

Materials and Methods

Normal human serum was used as a source of cholinesterase. *Cholinesterase activity* was determined at 37° C by electrometric titration at pH 7.4 in a medium consisting of 87 mM NaCl and 4.3 mM $MgCl_2$ with acetylcholine iodide and butyrylcholine bromide (4.3 mM) as substrates.

Determination of migration distance of cholinesterase in paper electrophoresis. An LKB electrophoresis equipment (LKB Fabriksaktiebolag, Stockholm) was used. The paper strips (SCHLEICHER & SCHÜLL no. 2043 bmgl) were wetted with the buffer (barbital pH 8.6, $\mu = 0.075$) and blotted on filter paper. To obtain comparable values, the conditions of electrophoresis were kept identical from experiment to experiment: The paper was equilibrated with the power (3.5 V/cm) switched on for 3 hours ($\pm$ 10 min) before the samples were applied, and the time of electrophoresis was 18 hours ($\pm$ 10 min). The spots of serum placed on the paper were small (1 μl) to reduce the error in determining the migration distance (distance from the point of application to the center of the spot). Electrophoresis was performed at room temperature.

Visualization of cholinesterase: The wet strips were immersed in a solution of 2 M NaCl, 50 mM NaH_2PO_4 adjusted to pH 7.3 with 1 N NaOH, 10 mM $MgCl_2$, 0.2 mg/ml α-naphthyl acetate and 0.2 mg/ml "Fast Blue B Salt" (diazonium salt of o-dianisidine, G. T. Gurr Ltd., London). In the course of 2—10 minutes at room temperature a red spot appeared on the paper at the site of the cholinesterase. The paper strips were finally rinsed in distilled water and dried (PINTÉR 1957).

Electrophoresis of serum protein: On the strips to be used for evaluation of the distribution of protein 20 μl of serum were applied as a narrow transversal band. After electrophoresis the strips were dried at 110° C to denature the protein and stained for 10 min in a saturated solution of "Amidoschwarz 10 B" in a mixture of 45 per cent (v/v) methanol, 45 per cent (v/v) water and 10 per cent (v/v) acetic acid. Then the strips were washed in several changes of methanol-water-acetic acid. The electropherogram was recorded on a paper strip scanner with logarithmic output.

Sialidase: The sialidase preparation used was "Receptor Destroying Enzyme" (lot no. 25, Behringwerke, Germany) which according to the manufacturer is a dried filtrate from cultures of Vibrion cholerae. Two ml of human serum dialyzed against distilled water and one ml of 50 mM NaH_2PO_4 adjusted to pH 6.8 with 1 N NaOH were incubated with 1—25 mg per ml serum of the sialidase preparation at 25° C for variable lengths of time. A drop of toluene was added to prevent bacterial contamination. A sample of buffered serum kept for the same length of time but without sialidase served as a control.

Chromatography of serum: Serum was chromatographed on diethylaminoethyl-cellulose (DEAE-cellulose, Whatman DE 50). One ml of serum dialyzed against 5 mM NaH_2PO_4 adjusted to pH 7.0 with 1 N NaOH was applied to a column (10 $\times$ 120 mm) and eluted with a decreasing pH and increasing salt gradient from 5 mM phosphate buffer to 0.5 M NaCl and 50 mM NaH_2PO_4 (SOBER et al. 1956). The eluate was collected in 5 ml fractions; protein was determined by measuring the transmittance at 280 mμ (BECKMAN spectrophotometer, model DU) and the cholinesterase activity was assayed by the semi-quantitative procedure described above: 10 μl spots were applied to filter paper and the

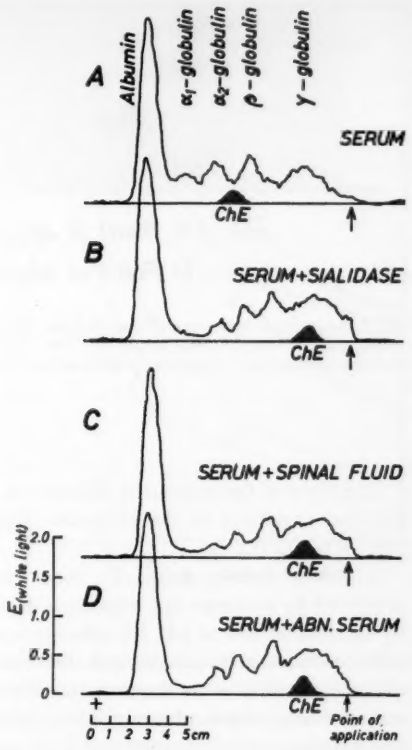

Fig. 1. *The effect of sialidase on the mobility of cholinesterase.* Paper electrophoresis at pH 8.6 of (A) serum, (B) serum + sialidase (10 mg/ml), (C) serum + spinal fluid concentrated 100 times by ultrafiltration (50 µl/ml) and (D) serum + abnormal serum (20 µl/ml). The incubation time was 10 days at 25° C. One paper strip was stained for protein and another for cholinesterase. Black areas indicate the localization of cholinesterase activity (ChE).

paper immersed in the α-naphthyl acetate - "Fast Blue B Salt" solution. The intensity of the red color indicated the cholinesterase activity. By this method 100 samples could be assayed in less than half an hour. As a control of the applied pH and salt gradient the conductivity and pH of the effluent were determined (Conductivity Meter CDM 2 and pH Meter PHM 22, Radiometer, Copenhagen).

Results

Effect of sialidase on cholinesterase.

1. *Change of mobility.* After incubation at pH 6.8 with sialidase the mobility of cholinesterase decreased with time until the mobility corresponded to that of the slowest part of the γ-globulin. The mobilities of α- and β-globulins were reduced as well, although not to the same extent as the mobility of cholinesterase (Fig. 1 A and B). About one-twentieth of the protein was precipitated during incubation.

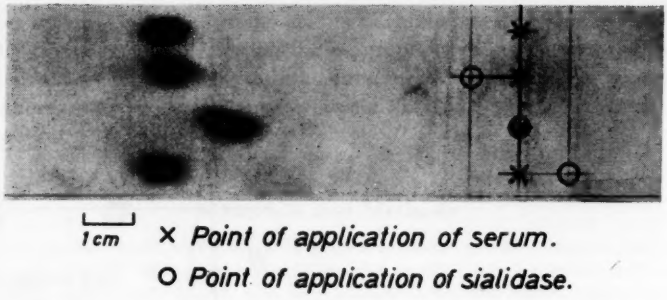

Fig. 2. Interaction between sialidase (0.1 mg per spot) and cholinesterase (2 μl serum per spot) during paper electrophoresis at pH 8.6. The strip was stained for cholinesterase. The mobility of cholinesterase was reduced only when serum and sialidase were applied together.

The ratio of the migration distance of the sialidase-treated cholinesterase (at minimal mobility) to the migration distance of untreated cholinesterase was 0.35 ± 0.005 (11 exp.).

2. *Rate of mobility change.* To investigate the rate of change of the mobility produced by sialidase the migration distance of cholinesterase was determined by electrophoresis at pH 8.6 after various times of incubation. The period of incubation to be considered was the time from incubation to termination of the electrophoretic run as the reaction between sialidase and cholinesterase continued during electrophoresis. That sialidase and cholinesterase were not separated during electrophoresis was indicated by the following experiment:

Four spots of serum were applied along a transversal line on the paper strip and three spots of sialidase (0.1 mg in 2 μl) were placed in front of, superposed on and behind the serum spots (Fig. 2). Electrophoresis was performed for 18 hours at pH 8.6 and the strips were stained for cholinesterase. The mobility of cholinesterase was reduced when sialidase and serum were applied together. This indicates that the reaction between sialidase and cholinesterase continues during electrophoresis. Sialidase placed in front of or behind the serum spot did not interact with cholinesterase. Thus, the mobilities of sialidase and of cholinesterase were roughly identical and the reaction between the enzymes can proceed during at least part of the electrophoresis.

The ratio of migration distances of sialidase-treated to untreated cholinesterase was determined at various time intervals after incubation with 1—25 mg/ml of the sialidase preparation (Fig. 3). With a given sialidase concentration the mobility of cholinesterase decreased gradually with time. The rate of the mobility change increased with increasing concentration of sialidase and minimal mobility was only reached after more than six days of incubation.

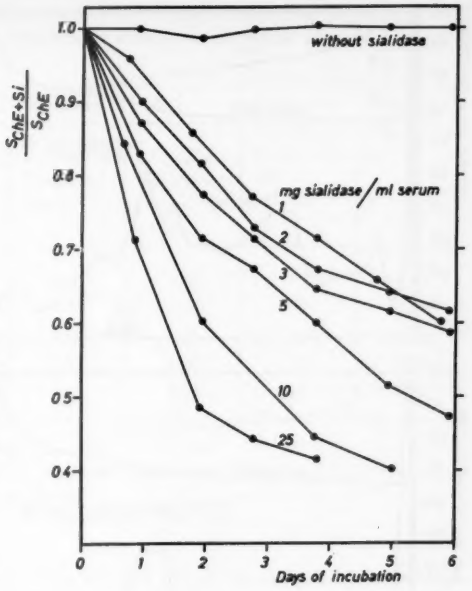

Fig. 3. Influence of sialidase on the mobility of cholinesterase as a function of incubation time. The ratio of migration distance of sialidase-treated and untreated cholinesterase (S_{ChE+Si}/S_{ChE}) was used as a measure of the mobility. Human sera were incubated at 25° C with 1—25 mg/ml sialidase at pH 6.8 and the migration distance determined by paper electrophoresis at pH 8.6. The curve for 5 mg/ml is averaged from 4 experiments and those for 10 and 25 mg/ml from 2 experiments.

3. *Effect of sialidase on the DEAE-cellulose chromatogram of cholinesterase.* The decrease in acidity of cholinesterase after treatment with sialidase was also evident from a comparison of the DEAE-cellulose chromatograms of sialidase-treated and untreated human sera (Fig. 4). The sialidase-treated cholinesterase was eluted together with neutral protein (γ-globulin) while the untreated cholinesterase appeared after albumin.

4. *Effect of sialidase on the enzymatic activity of cholinesterase.* The appearance of a red color in the visualization procedure for cholinesterase (see p. 268) indicates that the enzyme can still split α-naphthyl acetate after treatment with sialidase. A quantitative expression of the enzymatic activity of cholinesterase was obtained by electrometric titration with acetylcholine and butyrylcholine as substrates. The cholinesterase activity remained unchanged after treatment with sialidase (Table 1).

Occurrence of sialidase in human serum and spinal fluid

Human serum normally did not exhibit detectable sialidase activity. This was indicated by the identical mobility of cholinesterase in 50 serum samples immediately after withdrawal and after keeping at room temperature for up to two months.

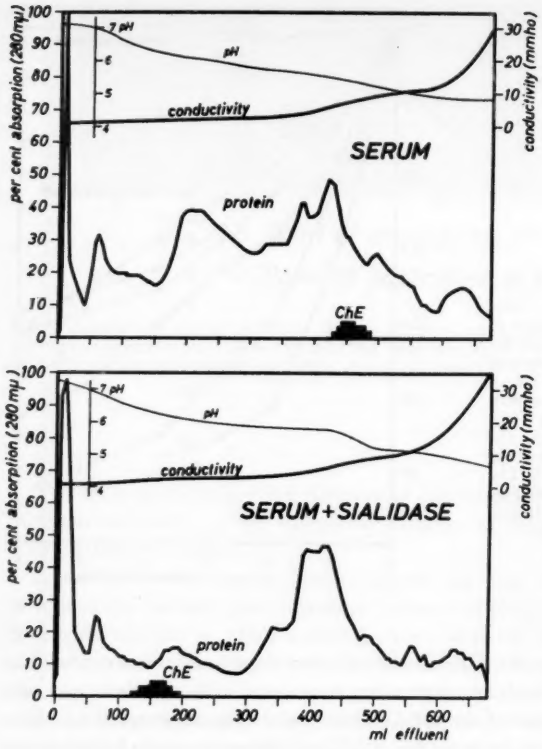

Fig. 4. Chromatography of untreated and sialidase-treated human serum on DEAE-cellulose columns. Serum was incubated at 25° C with 10 mg/ml sialidase for 10 days. The heights of the black columns indicate the activities of cholinesterase (ChE) as determined by the semiquantitative spot-assay. The upper curves indicate the pH and conductivity of the effluent.

Spinal fluid, on the other hand, changed the mobility of human serum cholinesterase and of α- and β-globulins in the same way as the sialidase preparation (Fig. 1 C). This effect was found in spinal fluid from three different pools with an average protein content of 0.38 mg/ml and normal electropherograms. In seven experiments the minimal ratio of migration distance of spinal fluid-treated and untreated cholinesterase was 0.34 ± 0.007, i. e. the same as obtained with the sialidase preparation. The activity as estimated from the effect on the mobility of cholinesterase was low: One ml of spinal fluid corresponded to 0.1 mg of the sialidase preparation.

In a single serum pool from hospitalized patients a reduced mobility of cholinesterase was observed. The mobilities of α- and β-globulin were also decreased. The ratio of the migration distance of cholinesterase from this pool to that of cholinesterase from all other sera investigated was 0.34, i. e. the same order as found for sialidase-treated cholinesterase. Addition of small amounts

Table I. Absence of an effect of a sialidase preparation (10 mg/ml serum) on the enzymatic activity of human serum cholinesterase. Cholinesterase activity was determined titrimetrically at pH 7.4 and 37° C

Serum	Cholinesterase activity [μmoles/(min. ml)][1]				Days of incubation at 25° C and pH 6.8	$\frac{S_{ChE+Si}}{S_{ChE}}$ [2]
	Acetylcholine		Butyrylcholine			
	Untreated serum	Sialidase-treated serum	Untreated serum	Sialidase-treated serum		
1	3.10	2.94	6.24	6.56	8	0.34
1	3.10	3.12	6.36	6.30	60	0.34
2	3.82	3.88	7.88	7.85	8	0.36
2	3.97	3.85	7.92	8.00	9	0.36
3	3.95	3.92	8.36	8.25	11	0.36

[1] duplicate or triplicate determinations.

[2] The ratio of migration distances of sialidase-treated and untreated cholinesterase as determined by paper electrophoresis at pH 8.6.

of the abnormal serum to normal serum (e. g. 1 μl/ml) produced similar reduction in the mobilities of cholinesterase and of α- and β-globulin as obtained with sialidase. The ratio of migration distances of cholinesterase after and before incubation with the abnormal serum was 0.34 ± 0.003 (20 exp.). The electropherogram of serum incubated with the abnormal serum was the same as after incubation with sialidase (Fig. 1 D). The effect of the abnormal serum was abolished by heating to 90° C for 5 min whereas dialysis had no influence on the effect. These findings indicate the presence of sialidase in the abnormal serum sample. The effect of 1 μl of the abnormal serum on the mobility of cholinesterase corresponded approximately to the effect of 1 mg of the sialidase preparation. Unfortunately it has not been possible to find the donor of this serum.

Discussion

The findings reported in this study suggest that the acidity of human serum cholinesterase is due to the presence of sialic acid bound to the protein in such a way that its release by sialidase does not expose charged sites. Although the number of sialic acid residues could not be estimated, the gradual decrease of mobility of cholinesterase during incubation with sialidase which proceeds without distinct steps, indicates that several molecules of sialic acid are involved. The isoelectric point of normal cholinesterase was displaced by treatment with sialidase from less than 3 to about 7 (SVENSMARK and KRISTENSEN, unpublished). A similar decrease in mobility has been described for acid α_1-glycoprotein after

treatment with sialidase (POPENOE and DREW 1957). Fifteen to sixteen molecules of sialic acid per molecule of this protein are released with a simultaneous increase of the isoelectric point from 2.7 to 5.0. Similar effects of sialidase on other serum sialoproteins (transferrin, orosomucoid, haptoglobin and coeruloplasmin) have been observed (ONCLEY, EYLAR and SCHMID 1958, LAURELL and BRÖNNESTAM 1959) and the effect on the α- and β-globulins of serum has been described by SCHULTZE and SCHWICK (1957) and by LAURELL (1959).

It is remarkable that the enzymatic activity of cholinesterase is retained after removal of acid residues from the molecule, i. e. under the conditions investigated a substantial change in charge did not affect the enzymatic activity. These findings are in contrast to the inactivation of "Follicle Stimulating Hormone" and human "Menopausal Gonadotropin" by enzymic release of N-acetylneuraminic acid and glycolyl-neuraminic acid respectively (GOTTSCHALK, WHITTEN and GRAHAM, 1960, GOT and BOURRILLON 1961).

That sialidase activity could not normally be detected in human serum is in agreement with the observation by Warren and Spearing (1960). They found, however, a weak sialidase activity in commercial bovine and human plasma fractions (α- and β-globulins). Provided that our three pools of spinal fluid were not contaminated by abnormal spinal fluid, sialidase is a normal constituent of spinal fluid. This is also suggested by the occurrence of free sialic acid in spinal fluid (UZMAN and RUMLEY 1956). Similarly, the occurrence of a cholinesterase fraction with a mobility comparable to that of γ-globulin in spinal fluid (SVENSMARK 1958, 1960, 1961) indicates the presence of sialidase.

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Urinary Excretion Patterns for Substances with Simultaneous Secretion and Reabsorption by Active Transport

By

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Abstract

BERGLUND, F. *Urinary excretion patterns for substances with simultaneous secretion and reabsorption by active transport.* Acta physiol. scand. 1961. *52.* 276—290. — Substances, which are actively secreted and reabsorbed by the renal tubules, show different excretion patterns. Except for potassium, they all follow a "secretion proximal" pattern, i. e. secretion takes place at a site proximal to reabsorption or through identical cells. Thiosulfate, creatinine and uric acid are typical examples. — For *thiosulfate*, secretion Tm = reabsorption Tm. At low plasma levels its clearance is low; at higher levels the clearance equals glomerular filtration rate. — For *creatinine*, secretion Tm > reabsorption Tm; its clearance is low at low plasma levels, but exceeds glomerular filtration rate at high levels. The dependence of "endogenous creatinine clearance" on glomerular filtration rate and renal blood flow is discussed. — A similar pattern is shown by *uric acid* in the rabbit. In man, uric acid reabsorption Tm > secretion Tm, the net result being reabsorption at all plasma levels.

The formation of urine comprises three main processes, namely 1. ultrafiltration of plasma in the glomeruli; 2. tubular reabsorption of solutes and water; 3. tubular secretion of solutes. Reabsorption and secretion by the tubules are effected by "active transport" and/or diffusion.

"Active transport" by the renal tubules is usually supposed to occur in one direction only, *i. e.* either as reabsorption or secretion. There are, however, substances which are both reabsorbed and secreted in certain species, *e. g.* thiosulfate, creatinine, uric acid and potassium. On the basis of experiments

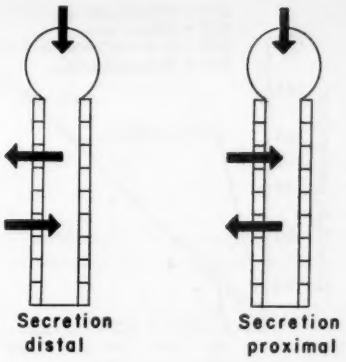

Fig. 1. Relative localization of secretion and reabsorption in the renal tubules.
"Secretion distal": solute is reabsorbed proximally, and secreted at a more distal site.
"Secretion proximal": solute is secreted proximally, and reabsorbed at a more distal site, or at the same site as secretion.
"Distal" and "proximal" do not signify localization to distal or proximal tubules.

with thiosulfate, BERGLUND, HELANDER and HOWE (1960) have formulated a "secretion proximal" and "secretion distal" concept. The proofs relating to "secretion proximal" are partly new, but most originate from papers published during the last two decades, though not fully interpreted at the time. Often the proof has been of pharmacological nature; inhibition of secretion has revealed the existence of simultaneous secretion and reabsorption (as for thiosulfate, creatinine and uric acid). The following presentation shows that "secretion proximal" often gives rise to characteristic excretion or clearance patterns.

Relative sites of secretion and reabsorption

Depending on the sites of secretion and reabsorption, relative to each other, a "secretion distal" and a "secretion proximal" pattern may be distinguished (Fig. 1). "*Secretion distal*" involves: filtration in the glomeruli, reabsorption at a proximal site in the tubules, and finally secretion at a more distal site. Everything secreted appears in the urine.

A substance following the "*secretion proximal*" pattern is filtered in the glomeruli and secreted at a proximal site in the tubules. Reabsorption takes place at a more distal site. Both filtered and secreted substance may thus be reabsorbed. — If a substance is secreted and reabsorbed at identical sites in the tubules, any amount secreted will immediately be available for reabsorption. This alternative is therefore included in "secretion proximal".

The terms "secretion distal" and "secretion proximal" should not be interpreted in terms of localization to the distal or proximal tubules; they only signify the site of secretion relative to reabsorption.

Relative magnitudes of transfer maxima (Tm's)

The shape of the excretion and clearance curves depends on the relative magnitudes of reabsorption Tm and secretion Tm. The first condition to be

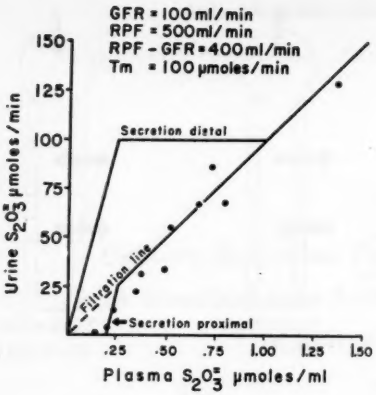

Fig. 2. Urinary excretion of thiosulfate in female dog, with GFR around 90 ml/min. Each ● is average of three clearance periods, adjusted to GFR 100 ml/min. — Theoretical lines show expected urine thiosulfate for "filtration", "secretion distal" and "secretion proximal". For assumptions and calculations, see Table I. Modified from BERGLUND, HELANDER & HOWE (1960).

discussed is that of equal Tm's in both directions (section I). This section illustrates the difference between "secretion distal" and "secretion proximal". Sections II and III describe cases with unequal Tm's for secretion and reabsorption, but only for substances with "secretion proximal". Potassium is therefore not included.

In the calculations which follow it has been assumed that both secretion and reabsorption show typical Tm curves, *i. e.* the substance is completely extracted from peritubular blood or from tubular urine until Tm is reached.

I. Reabsorption Tm = Secretion Tm. Example: Thiosulfate in man and female dog.

In man (BUCHT 1949) and dog (FOULKS *et al.* 1952) thiosulfate is both secreted and reabsorbed. In the female dog the Tm in each direction is around 125 µmoles/100 ml glomerular filtrate (BERGLUND *et al.* 1960). Fig. 2 shows expected urinary excretion of thiosulfate, assuming filtration only, or "secretion distal" or "secretion proximal". The figure is based on the assumptions and calculations in Table I.

In the "*secretion distal*" pattern filtered thiosulfate would be completely reabsorbed at low plasma levels. Secreted thiosulfate would appear in the urine and soon reach a Tm. With further increasing plasma levels, filtration and reabsorption of thiosulfate increase together. When reabsorption Tm is reached, filtered thiosulfate would appear in the urine, and the excretion would then follow the "filtration line".

If thiosulfate followed the "*secretion proximal*" pattern, both filtered and secreted thiosulfate would be reabsorbed at low plasma levels. When reabsorption Tm is reached, the excretion would rise steeply because of addition both from glomerular filtration and tubular secretion. When secretion Tm is reached, urinary excretion would follow the "filtration line".

Table I. Expected urinary excretion of thiosulfate at selected plasma levels

Modified from BERGLUND, HELANDER and HOWE (1960).

Assume: Reabsorption Tm = Secretion Tm = 100 μmoles/min.
GFR = 100 ml/min.
RPF = 500 ml/min.
RPF — GFR = 500 — 100 = 400 ml/min.
Then: Filtered S_2O_3 = 100 × Plasma S_2O_3
Secreted S_2O_3 = 400 × Plasma S_2O_3
"Secretion distal": Urine S_2O_3 = (Filtered S_2O_3 — Reabsorbed S_2O_3) + Secreted S_2O_3.
"Secretion proximal": Urine S_2O_3 = (Filtered S_2O_3 + Secreted S_2O_3) — Reabsorbed S_2O_3.

Plasma thiosulfate	Urine thiosulfate	
	"Secretion distal"	"Secretion proximal"
μmoles/ml	μmoles/min.	μmoles/min.
0.15	(15 — 15) + 60 = 60	(15 + 60) — 75 = 0
0.20	(20 — 20) + 80 = 80	(20 + 80) — 100 = 0
0.25	(25 — 25) + 100 = 100	(25 + 100) — 100 = 25
1.00	(100 — 100) + 100 = 100	(100 + 100) — 100 = 100
1.25	(125 — 100) + 100 = 125	(125 + 100) — 100 = 125

It is of interest to compare "secretion distal" and "secretion proximal". Secretion becomes saturated at the same plasma level (0.25 μmoles/ml) in both cases. Reabsorption becomes saturated at high plasma levels in "secretion distal" (only filtered load is reabsorbed), but at low plasma levels in "secretion proximal" (filtered + secreted load is reabsorbed). The "filtration line" is intercepted at different plasma levels, namely when both secretion and reabsorption become saturated.

Urinary excretion data in the dog (Fig. 2) show that thiosulfate follows the "secretion proximal" pattern (BERGLUND *et al.* 1960). Data from competition studies further indicate that secretion and reabsorption are effected by identical cells.

II. "Secretion proximal". Secretion Tm > Reabsorption Tm. Examples: Creatinine in man, rat, goat. Uric acid in rabbit. PAH and diodrast in Necturus.

This alternative shows a characteristic excretion pattern. At low plasma levels the clearance is low, sometimes below glomerular filtration rate (GFR). As the plasma level increases, the clearance first rises above and then again approaches GFR.

Creatinine follows such a pattern in the goat (Fig. 3, reconstructed from LADD *et al.* 1957). At a plasma level of 1 mg % its clearance equals inulin clearance. At 3 mg % the clearance is twice as high, and then gradually decreases. Probenecid, p-(dipropylsulfamyl)-benzoic acid, partly inhibits the secretion. —

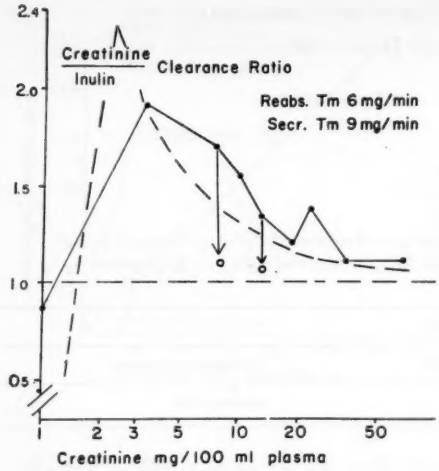

Fig. 3. ●—●—● creatinine clearance in the goat; ○ ○ after probenecid. Reconstructed from LADD et al. (1957). — — — theoretical clearance curve, cf. Table II.

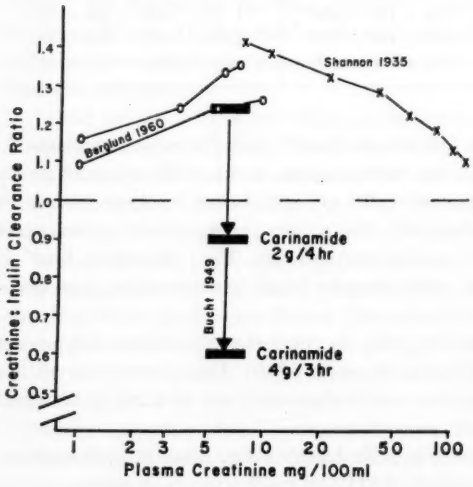

Fig. 4. Creatinine clearance in man. Maximum creatinine/inulin clearance ratio (1.3—1.4) around plasma level of 8—10 mg %. Carinamide® depresses clearance ratio below unity. — Constructed from data of SHANNON (1935), BUCHT (1949) and two own experiments ("Berglund 1960"). — Technical data on "Berglund 1960": Two identical twins. Each ○ represents three 10-minute periods. Water diuresis 10—14 ml/min. Inulin clearance 118 and 119 ml/min respectively. Creatinine analysis according to LØKEN (1954), inulin according to HEYROVSKY (1956).

Table II shows expected clearances of creatinine, for GFR = 100 ml/min, reabsorption Tm = 6 mg/min and secretion Tm = 9 mg/min. The Tm values were chosen by trial and error. The theoretical curve shows fair agreement with the experimental data (Fig. 3).

In man the clearance follows a flatter curve than in the goat (Fig. 4). The maximum creatinine/inulin clearance ratio is only 1.4 and is reached at a plasma level as high as 8—10 mg %. On the other hand it is possible to block

Table II. Expected urinary excretion and clearance of creatinine at selected plasma levels

Assume: Reabsorption Tm = 6 mg/min.
Secretion Tm = 9 mg/min.
GFR = 100 ml/min.
RPF = 500 ml/min.
RPF — GFR = 500 — 100 = 400 ml/min.
Urine creatinine: (Filtered + Secreted) — Reabsorbed.

Plasma creatinine	Urine creatinine	Clearance
mg/100 ml	mg/min.	ml/min.
1.2	1.2 + 4.8 — 6 = 0	0
1.5	1.5 + 6 — 6 = 1.5	100
2.25	2.25 + 9 — 6 = 5.25	233
3	3 + 9 — 6 = 6	200
5	5 + 9 — 6 = 8	160
10	10 + 9 — 6 = 13	130
	Varying glomerular filtration rate	
Assume: GFR = 50 ml/min. RPF = 500 ml/min. RPF — GFR = 450 ml/min.		
1.5	0.75 + 6.75 — 6 = 1.5	100
Assume: GFR = 200 ml/min. RPF = 500 ml/min. RPF — GFR = 300 ml/min.		
1.5	3.0 + 4.5 — 6 = 1.5	100
	Varying renal plasma flow	
Assume: GFR = 100 ml/min. RPF = 450 ml/min. RPF — GFR = 350 ml/min.		
1.5	1.5 + 5.25 — 6 = 0.75	50
Assume: GFR = 100 ml/min. RPF = 450 ml/min. RPF — GFR = 450 ml/min.		
1.5	1.5 + 6.75 — 6 = 2.25	150

the secretion so completely (or selectively?) with carinamide®, p-(benzylsulfonamido)benzoic acid, that reabsorption dominates.

In the white rat and in the kangaroo rat similar conditions prevail (Table III). "Endogenous creatinine clearance" is practically identical with the inulin clearance; at elevated levels of creatinine, its clearance is almost twice as high. Probenecid depresses the creatinine/inulin clearance ratio towards unity.

Dependence of creatinine clearance on glomerular filtration rate and renal blood flow. "Endogenous creatinine clearance" is often used as an approximate measure of GFR, on the assumption that creatinine at normal plasma levels is filtered only. As it, however, is also secreted and reabsorbed, its clearance may not always equal GFR. Theoretically the excretion and clearance of "endogenous

Table III. Creatinine/inulin clearance ratios at various plasma levels of creatinine, in unanesthetized rats and kangaroo rats

Species	Plasma Creatinine mg/100 ml	Creatinine/Inulin clearance ratio	Investigator
Rat	[1]0.29 ± 0.01 22.3 ± 1.0 (Means ± standard error)	1.10 1.98 ± 0.22	PETERS (1959)
Rat	20 → 150	1.90 → 1.35	FINGL (1952)
	Highest ratios at low plasma levels. Ratios depressed towards 1.0 by probenecid		
Kangaroo rat (*Dipodomys merriami*)	[1]0.3 1.6 4 11 31	1.1 (?) 1.0 1.3 1.7 1.4	SCHMIDT-NIELSEN (1954)

[1] "Endogenous creatinine"

creatinine" should actually be independent of GFR, because any increase of the amount filtered will be counterbalanced by a reduction of the creatinine available for tubular secretion, and *vice versa* (Table II). This is illustrated by the two cases in Table IV, in which GFR was increased by oxyphyllin®

Table IV. Effect of oxyphyllin® on inulin and PAH clearances, and on the excretion of endogenous creatinine. From tables 16 and 17 in EK *(1955)*

	Clearance		Urine creatinine mg/min.
	Inulin ml/min.	PAH ml/min.	
	Normal A6		
Control	166	801	1.11
	158	834	1.09
	186	969	1.30
Oxyphyllin 1.35 g i. v.	357	1 002	1.47
	347	911	1.27
	Hypertension Hyp 14		
Control	141	462	1.22
	130	399	1.33
	130	391	1.25
Oxyphyllin 1.35 g i. v.	309	431	1.51
	266	384	1.33

Table V. Effect of nepresolin® on clearances in the goat

Dose of nepresolin	Plasma creatinine	Clearance			Ratio Creatinine/ Inulin Clearance
		PAH	Inulin	Creatinine	
	mg/100 ml	ml/min.	ml/min.	ml/min.	
Exp. 6					
0	0.99	232	48	56	1.15
25 mg i. v.	0.91	410	63	79	1.26
25 mg i. v.	1.08	456	54	66	1.19
Exp. 7					
0	1.07	336	68	71	1.05
25 mg i. v.	0.99	559	58	72	1.29

Female goats, about 40 kg body weight. The figures are average of 3, 3 and 2 clearance periods in exp. 6; 3 and 5 periods in exp. 7.

(theophyllin-1-aminopropanol) (Ek 1955). After injection of oxyphyllin the inulin clearance doubled, PAH clearance remained unchanged, whereas the excretion of "endogenous creatinine" increased by less than 20 %, in fair agreement with the calculations in Table II.

In contrast, the "endogenous creatinine clearance" should be highly sensitive to changes in renal blood flow, so that a 10 % change in renal plasma flow (RPF) should change the "endogenous creatinine clearance" by 50 % (Table II). To test the sensitivity to changes in RPF, experiments were done with nepresolin® (Ciba; 1,4-dihydrazinophtalazine methane sulfonate) in goats (Table V). In two experiments PAH clearance doubled, whereas inulin clearance increased or decreased slightly; the creatinine/inulin clearance ratio increased in both experiments, but much less than expected. This might be due to less complete extraction of creatinine from the peritubular capillaries, when renal blood flow increased above normal values.

Thus, "endogenous creatinine clearance" does not rise significantly when GFR *or* RPF is increased. A marked rise occurs, however, when GFR and RPF increase together (Bucht *et al.* 1956). This would be expected, because filtered creatinine then increases without reducing the amount available for secretion by the tubules.

When GFR and RPF fall below normal, as in renal insufficiency, the "endogenous creatinine"/inulin clearance ratio is usually elevated and may reach values as high as 2.2 (Mattas *et al.* 1952); this might partly be due to an increased plasma level, but selective impairment of the reabsorption mechanism cannot be excluded.

When GFR is strongly depressed by circulatory disturbances, as occurs in man in diabetic coma or in morphine-scopolamine intoxication, the "exogenous

Table VI. Creatinine/Inulin clearance ratios with falling plasma levels of creatinine

Time	Plasma creatinine	Clearances		Ratio Creatinine/ Inulin Clearance
		Inulin	Creatinine	
min.	mg %	ml/min.	ml/min.	
	EXP. 14			
0	Prime: inulin 4 g, creatinine 6 g. Infusion: inulin 0.06 g/min, creatinine 0.06 g/min, Na_2SO_4 0.09 g/min. in H_2O 1,3 ml/min.			
31—60	36	96	106	1.14
137—156	39	97	112	1.16
157	Creatinine discontinued			
280—309	12	88	117	1.33
	EXP. 15			
	4 liters H_2O at — 30 min. and at 168 min.			
0	Prime and Infusion = Exp. 14, without Na_2SO_4			
30—51	42	82	87	1.07
140—159	45	73	88	1.20
160	Creatinine discontinued			
285—310	15	84	116	1.39

Female goat, 40 kg, 3 weeks after pregnancy. In exp. 14 osmotic diuresis (1.8—3.8 ml/min.). In exp. 15 water diuresis (9—13 ml/min.), with hemolysis and hemoglobinuria during last hour. Each figure is average of 2—3 periods, of 7—10 minutes duration.

creatinine clearance" falls below the inulin clearance (McCance and Widdowson 1939). This has been ascribed to increased tubular permeability, permitting backdiffusion of creatinine. Tubular secretion may, however, still persist. Thus, in the goat restriction of blood flow to one kidney decreased the creatinine/inulin clearance ratio from 1.5 to 0.75. Probenecid depressed this clearance ratio still further (to < 0.3), evidently by blocking secretion (Ladd *et al.* 1957).

Creatinine clearance with falling plasma levels. When the plasma level of creatinine is allowed to fall from high levels, the creatinine/inulin clearance ratio does not always rise as expected (Shannon 1935, Shannon and Ranges 1941, Ladd *et al.* 1957). In the goat, Ladd *et al.* obtained a ratio of 1.11 at a plasma level of 35 mg %, and 3 hours later a ratio of 0.64 at a plasma level of 12 mg %, reabsorption thus dominating over secretion. To further elucidate this, experiments were done in a female goat (Table VI). The plasma level was kept well above 30 mg % for 2 hours and then allowed to fall. The clearance ratio then rose above 1.3, instead of falling below 1.0. This was true both with osmotic diuresis and water diuresis. — The experiments of Shannon and Ranges (1941) were of a different character. They showed, in man, that a second dose of creatinine elevated the depressed clearance ratio. Unfortunately

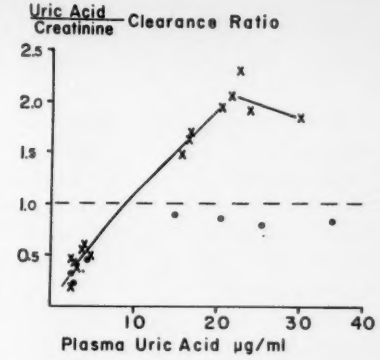

Fig. 5. Uric acid clearance in the rabbit (reconstructed from POULSEN 1955). × × without probenecid, ○ ○ after probenecid 50—150 mg/kg body weight intravenously.

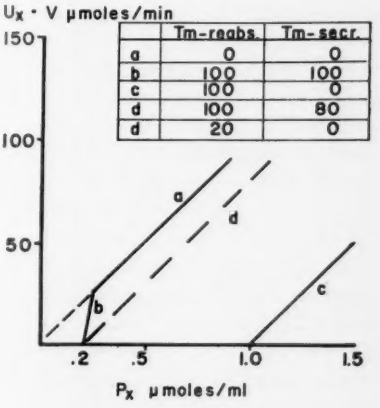

Fig. 6. Urinary excretion of substance X, with "secretion proximal", and reabsorption Tm > secretion Tm. GFR = 100 ml/min, RPF = 500 ml/min. $U_X \cdot V$ = urinary excretion. Note two possible interpretations of line *d*.

their plasma levels before the second dose were as low as 4—9 mg %, and after injection 14—21 mg %, so a higher clearance ratio was to be expected in most experiments (*cf.* Fig. 4). — It is concluded that depression of creatinine/inulin clearance ratios with falling plasma levels is not reproducible and is of doubtful significance.

Uric acid: In the rabbit, the clearance of uric acid follows a similar pattern. At low plasma levels, the uric acid/creatinine clearance ratio is well below 1.0 (Fig. 5). At higher plasma levels this ratio reaches a value of 2.3, but is depressed below unity by probenecid (POULSEN 1955).

PAH and Diodrast: In Necturus p-aminohippurate (PAH) and diodrast show net reabsorption at low plasma levels. At higher levels their clearances approach or exceed creatinine clearance (KINTER 1959). The pattern fits with "secretion proximal". — Autoradiographic studies in Necturus with dio-

drast-I^{131} show that the cells of the proximal tubules are the exclusive site of intracellular accumulation, and they appear to be the major site of diodrast transport, irrespective of the direction (KINTER, LEAPE and COHEN 1960).

III. "Secretion proximal". Reabsorption Tm > Secretion Tm. Example: uric acid in man.

This alternative is analyzed in Fig. 6. Lines *a* and *ba* are identical with "filtration line" and "secretion proximal" in Fig. 2. Line *c* (parallel to *a*) shows expected urinary excretion when reabsorption Tm = 100 μmoles/min and secretion Tm = 0. The reabsorption Tm would be reached at a plasma level of 1 μmole/ml.

For comparison, return to curve *ba:* reabsorption Tm = secretion Tm = 100 μmoles/min. If the secretion Tm is gradually diminished, line *a* will move to the right and soon originate directly from the abscissa (line *d*); this occurs at a plasma level of 0.2 μmoles/ml. At this point secretion Tm (now 80 μmoles/min) and reabsorption Tm (still 100 μmoles/min) will be reached simultaneously, and line *b* disappears. An identical pattern (line *d*) would be obtained if there were reabsorption alone with a Tm = 20 μmoles/min. A typical reabsorption Tm pattern therefore does not exclude tubular secretion of considerable magnitude.

This may be exemplified by uric acid, which in man is reabsorbed by the tubules. Uric acid appears in the urine before the "Tm" is reached, and at normal plasma levels about 10 % of "filtered uric acid" (filtered + secreted uric acid) escapes reabsorption. YÜ and GUTMAN (1955) reported that uricosuric drugs (sodium salicylate, phenylbutazone, probenecid) in therapeutic doses raise, but in smaller doses lower the excretion of uric acid. They suggested that salicylates etc. increase the net reabsorption of uric acid by inhibiting tubular secretion, and that uric acid, like potassium, is reabsorbed proximally and secreted distally. The excretion pattern described above suggests, however, a "secretion proximal" pattern similar to that in the rabbit, but with reabsorption dominating at all plasma levels.

Comparative physiology

Simultaneous secretion and reabsorption following the "secretion proximal" pattern has been demonstrated for thiosulfate, uric acid, creatinine, PAH and diodrast. Both secretion and reabsorption are probably effected by the proximal tubules, as with diodrast in Necturus. The two-way transport may originate from secretory systems occurring among the vertebrates, complete reversal yielding reabsorption, partial reversal yielding secretion + reabsorption.

This may be exemplified by the transport system for *sulfate* and *thiosulfate* (Table VII) (BERGLUND and FORSTER 1958, BERGLUND *et al.* 1960, BUCHT 1949). Both ions are secreted by the aglomerular kidneys of the Lophius. In the dog and in man, sulfate is reabsorbed, while thiosulfate is both secreted

Table VII. Comparative physiology of substances which follow a "secretion proximal" pattern

Substances	Species	*Secretion.* (+) Inhibitors	*Reabsorption.* (+) Inhibitors
Thiosulfate Sulfate	Goosefish *(Lophius americanus)*	+ Mutual inhibition Carinamide	
	Dog Man	*Thiosulfate only* + Sulfate Carinamide	*Both ions* + Mutual inhibition
Diodrast PAH	Rabbit Man	+ Mutual inhibition	
	Salamander *(Necturus maculosis)*	+ Mutual inhibition	+ Mutual inhibition
Creatinine	Marine fish Bullfrog Chicken Anthropoid apes	+ Phlorizin	
	Rat Goat Man	++ Phlorizin	+
Uric acid	Lizard *(Iguana iguana* Shaw*)*	+	
	Chicken	+ Probenecid Salicylates	
	Rabbit	++ Probenecid Salicylates	+
	Dog, non-dalmatian		+ Probenecid
	Dog, Dalmatian	++ Probenecid	+ (?)
	Man	+ Probenecid Salicylates	++ Probenecid Salicylates

+ means occurrence of active transport (secretion or reabsorption). ++ signifies the higher Tm in case of bidirectional transport. With secretion ++ and reabsorption +, reabsorption may still predominate at low plasma levels (cf. Fig. 5). Absence of + means that transport has not been demonstrated.

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and reabsorbed. Still, the two ions seem to be transported by the same carrier. They inhibit each other's transport competitively, both in the direction of reabsorption and secretion. Carinamide®, but not probenecid, inhibits the secretion of both ions.

Similar reciprocal inhibition occurs between *diodrast* and *PAH* (Table VII). They have been shown to inhibit each other's secretion in rabbit and man (JOSEPHSON *et al.* 1953 b and a). In Necturus they are both secreted and reabsorbed and mutually inhibit each other's transport in both directions (KINTER 1959).

Creatinine is secreted by the renal tubules in frog (SWANSON 1956), marine fish, chicken and anthropoid apes (for ref. see SMITH 1951, p. 143). It is both secreted and reabsorbed in rat, goat and man, and in these species secretion is inhibited by carinamide® (BUCHT 1949) or probenecid (FINGL 1952, LADD *et al.* 1957). These inhibitors have not been tested in fish, chicken or frog, but most likely the secretion is effected by the same mechanism in all vertebrates involved. Phlorizin depresses the creatinine/inulin clearance ratio in the dogfish, frog, chicken, goat, chimpanzee and man (for ref. see SMITH 1951, pp. 98—99).

Uric acid. Uric acid is largely eliminated by tubular secretion in reptiles and birds, (for ref. see Smith 1951, p. 144).

Various excretion patterns exist in mammals. Uric acid is both secreted and reabsorbed in man, but the secretion is masked by the high reabsorption Tm. — In the rabbit reabsorption dominates at endogenous plasma levels (0.2—0.5 mg %), but secretion dominates at elevated plasma levels (Fig. 5). — In the dog uric acid is reabsorbed by the tubules. It is, however, secreted in the Dalmatian coach hound, in which the clearance may be twice as high as GFR (WOLFSON, COHN and SHORE 1950). Mixed breeds (Dalmatian — nondalmatian) may, like pure-bred dalmatians, be "high uric acid producers". TRIMBLE and KEELER (1938) noted that "Animals bearing white hairs diffused in their spots have been consistently 'low producers' of uric acid, while those animals which have had 'high uric acid production' have had uniform black pigment in their spots". Dalmatian hybrids studied by ONSLOW (1923) showed a similar trend. It may therefore be that "the recessive gene, which converts diffuse ticking or flecking to polka-dotting is the same gene which is responsible for the high uric acid excretion" (ALAN MITCHELL, personal communication 1956).

Secretion of uric acid is in general inhibited by probenecid and salicylates. Reabsorption, when present, may also be inhibited by these drugs (BEYER *et al.* 1951, POULSEN 1955, YÜ and GUTMAN 1955, NECHAY and NECHAY 1959, BERGER, YÜ and GUTMAN 1960). When both secretion and reabsorption are inhibited in man, uricosuria results.

The goat experiments were made possible by the helpful collaboration of Doctor BENGT ANDERSSON at Veterinärhögskolan, Stockholm. Doctor LARS OLBE performed one of the twin clearance experiments. Skilful technical assistance was given by Miss MONA HENRIKSSON.

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Augmented Cardiac Contraction, Heart Acceleration and Skeletal Muscle Vasodilatation Produced by Hypothalamic Stimulation in Cats

By

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Abstract

ROSÉN, A. *Augmented cardiac contraction, heart acceleration and skeletal muscle vasodilatation produced by hypothalamic stimulation in cats.* Acta physiol. scand. 1961. *52.* 291—308. — Hypothalamic stimulation in cats was found to produce an increase of the contractile force of the right ventricle as measured by a strain gauge arch. Concomitant tachycardia was generally observed. The responses were mediated both by cardiac sympathetic innervation and by catechols released from the adrenals. In some experiments the nervous mechanism dominated, in others the humoral factor. The hypothalamic area, the stimulation of which produced cardiac augmentation, appeared to correspond with "the sympathetic vasodilator area". Muscle vasodilator as well as cardiac augmentative responses appeared concurrently.

The sympathetic vasodilator nerves to the skeletal muscles in dogs and cats can be activated by hypothalamic stimulation. The vasodilatation produced during such stimulation is due to activation of cholinergic fibres and can be markedly reduced or abolished by atropinization of the animal. During central stimulation of the sympathetic vasodilator outflow vasomotor responses are not confined to the blood vessels in the muscles. Vasoconstriction occurs concomitantly in cutaneous and splanchnic regions (ELIASSON *et al.* 1951, LINDGREN and UVNÄS 1953, LINDGREN 1955), together with an increased adrenal release of catechols, mainly adrenaline (GRANT *et al.* 1958, LINDGREN, ROSÉN and UVNÄS 1959 a). Though the amounts of adrenaline released in most experiments are so minute as to cause only minor vascular effects, if any, they may nevertheless suffice to produce metabolic effects (GRANT *et al.* 1958, LINDGREN, ROSÉN and UVNÄS 1959 b).

The physiologic significance of the observed pattern of responses to intracerebral stimulation is not known. ELIASSON *et al.* (1951) and UVNÄS (1954) have suggested that activation of the sympathetic vasodilator outflow might occur in fear, anger, and other situations associated with emotional stress. Recent observations by ABRAHAMS and HILTON (1958), ABRAHAMS, HILTON and ZBRÓZYNA (1960 a and b) and BLAIR *et al.* (1959) have led to similar conclusions. Among other suggestions put forward is the possibility that the vasodilator system is in some way concerned with the initial adjustment of the muscular blood flow to exercise (UVNÄS 1954). The observed increase of muscle blood flow associated with posthaemorrhagic syncope led BARCROFT *et al.* (1944) and BARCROFT and EDHOLM (1945) to suggest that a sympathetically-mediated active vasodilatation occurred.

The aim of this paper is to contribute to elucidation of the functional significance of the sympathetic vasodilator system. It is sought to ascertain whether augmentation of cardiac functions such as contractile force and heart rate accompanies the characteristic peripheral responses to central stimulation of the vasodilator outflow. An affirmative finding would tend to support the above-mentioned hypothesis of Uvnäs concerning the functional significance of the sympathetic vasodilator system.

The literature contains few data on the influence on cardiac contractile force following hypothalamic stimulation. RUSHMER, SMITH and FRANKLIN (1959), and SMITH *et al.* (1960) localized in the canine hypothalamus a few small areas the stimulation of which caused changes in left ventricular performance identical with the responses to treadmill exercise. RUSHMER and collaborators consider that these observations lend weight to the old idea of a central nervous mechanism which causes an increase of cardiac work during the initial phase of muscle activity.

Many investigators have observed cardiac acceleration following hypothalamic stimulation, but few data on heart rate responses to hypothalamic stimulation of the sympathetic vasodilator pathway are found in the literature. In a few experiments in which the heart rate was studied, tachycardia was observed to accompany the muscle vasodilator response elicited by hypothalamic stimulation of the sympathetic vasodilator system (ELIASSON *et al.* 1951). No attempt was made, however, to investigate the mechanism behind the cardiac acceleration.

Method

Successful experiments were performed on 32 cats weighing 1.9 to 4.2 kg and anesthetized with chloralose (25—50 mg/kg) and urethane (200—500 mg/kg) i. v. About 80 cats were originally prepared, but technical difficulties encountered in attachment of the strain gauge arch to the heart, as well as in the maintenance of adequate cardiovascular conditions throughout the experiment necessitated elimination of a number of animals.

The trachea was cannulated. Arterial pressure was recorded by means of a plastic cannula inserted into one carotid artery with its tip in the aortic arch. The pressure was measured with a Statham transducer (P 23 A). Rectal temperature was maintained at about 37° C by means of an infrared lamp directed toward the animal's thorax and abdomen.

Blood flow was directed to a silicone-filled drop chamber (LINDGREN 1958), and via a photocell the drop rate was recorded by an ordinate writer. Muscle blood flow was determined in the right femoral artery in a skinned hind limb. For exclusion of paw blood flow a tight ligature was applied immediately above the ankle. To maintain warmth and moistness, the skin was replaced around the muscle. In 3 experiments the blood flow to an intestinal area was measured in a branch of the superior mesenteric artery. To prevent clotting, heparin (Heparin 5 %, Vitrum) 25 mg/kg was administered i. v.

In 11 of the experiments the cats were initially subjected to bilateral cervical vagotomy. In 3 of these, carotid occlusion was maintained to avoid possible cardiac changes due to baroceptor mechanisms. Dextran (Macrodex, Pharmacia) was given i. v., as required, to compensate for blood loss. Positive pressure artificial respiration was maintained during the experiments.

Central stimulation was effected by the Horsley-Clarke technique. With use of a dental drill the skull was trephined for insertion of the electrode into the right side of the hypothalamus. Electrical stimulation (2.0—2.5 V, 70 cps and pulse duration 2 msec with insulated stainless steel unipolar electrodes about 0.45 mm thick and with an uninsulated pointed tip 0.3 mm long) was produced by a square wave generator.

The two stellate ganglia were carefully located via a bilateral approach through the first costal interspace. The chest wall was then closed. Subsequently it was opened for removal of the ganglia and reclosed.

The two adrenals were dissected free via an abdominal approach and later ligated and removed. In some experiments, the pulmonary artery and either the abdominal aorta just below its passage through the diaphragm, or a thoracic part of the aorta, were mobilized and a heavy thread placed loosely around each of them for subsequent stricture.

The right intraventricular pressure was recorded in 11 experiments with a Statham transducer (P 23 A). Following thoracotomy a polyethylene catheter was advanced through the ventricular wall into the cavity, with the aid of a sharp metal guide which was then removed.

Heart rate was determined with an interval recorder (GOLDSCHMIDT and LINDGREN 1961) via impulses from the blood-pressure channel of a Grass (Polygraph) instrument.

Strain Gauge Arch Recording of Cardiac Contractile Force

Cardiac contractile force was measured by a strain gauge arch. In the following the terms "contractile force" and "force of cardiac contraction", insofar as they concern personal observations, denote the force of ventricular contraction as measured by a strain gauge arch.

After thoracotomy and opening of the pericardium the strain gauge arch was attached to the ventricular wall by sutures penetrating deeply into the muscle. All sutures were so placed as to avoid damage to or occlusion of any major branch of a coronary vessel. The muscle segment between the two points of attachment was conventionally stretched 40—50 per cent, as described by COTTEN and BAY (1956), to minimize errors in the recording of any subsequent increase in the size of the ventricle. The absence of irregularities in the cardiac recordings after attachment of the arch indicated that the conducting system of the heart had not been traumatized. Care was taken to shield the opening in

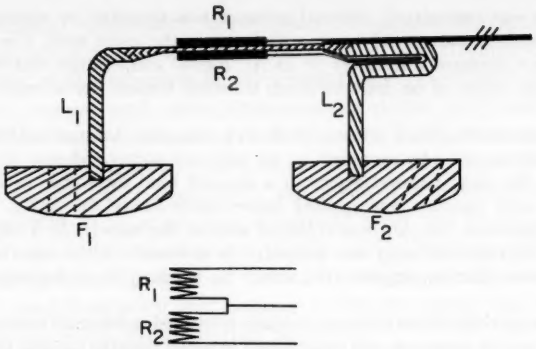

Fig. 1. Sagittal section of a modified strain gauge arch.
R_1 and R_2 The strain gauge resistances.
L_1 and L_2 The legs of the arch. The distance between L_1 and L_2 is 9 mm.
F_1 and F_2 The feet with holes for sutures.

the chest wall, thus preventing drying and cooling of the heart. The intrathoracic pressure was atmospheric during the experiment.

In all but one of the 20 experiments in which the hypothalamus was stimulated, strain gauge arch recording of contractile force was done from the right ventricle; in one, the arch was attached to the left ventricle. Right ventricular contractile force was also recorded in 12 supplementary experiments designed for qualitative testing of the strain gauge arch technique. In 8 of these experiments left ventricular force was studied simultaneously.

In 7 of the experiments in which the hypothalamus was stimulated, open strain gauge arches essentially similar to that reported by BONIFACE, BRODIE and WALTON (1953) were used. In the other experiments the strain gauge arch was slightly modified (Fig. 1), and resulted in a more sensitive and stable recording. Instead of one, two identical strain gauge wires (Philips PR 9214), each with a resistance of 120 ohms, were attached — one on either side of the metal (phosphor-bronze) strip connecting the two legs. With this arrangement, opposite changes occurred in the two resistances on application of a force between the two points of attachment of the arch: the resistance increased in the elongated wire and decreased in the contracted one. To ensure stable fixation and sufficient insulation of the strain gauge wires, the metal strip was lengthened on one side and attached to the subjacent part. By measuring the difference of the two resistances greater accuracy was achieved than by use of a single strain gauge wire (Fig. 2).

Supplementary Studies and Comments on the Qualifications of the Strain Gauge Arch Technique

In using the strain gauge arch technique for determination of cardiac contractile force, it is necessary to bear in mind that changes of heart size may cause interference. It is therefore essential in the present experiments to be on the alert for any rise of the heart volume due to elevated pressure in the aorta or to increased venous return.

Strain gauge arch recording of the left ventricular contractile force is influenced by the aortic pressure when the latter exceeds a certain level (Fig. 3). This level may be reached on intracerebral stimulation. The rise of left ventricular diastolic pressure secondary to that of the aortic diastolic pressure tends to increase the diastolic length of the muscle fibers, which thus contract more vigorously. Since the muscle segment between

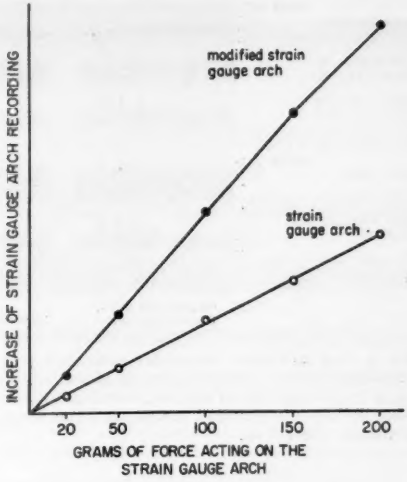

Fig. 2. Relationship between grams of force acting on the modified strain gauge arch and percentual increase of recorded force. Comparison with the sensitivity of the strain gauge arch described by Boniface *et al.* (1953). Under the experimental conditions the recorded force of the heart never exceeded 150 g.

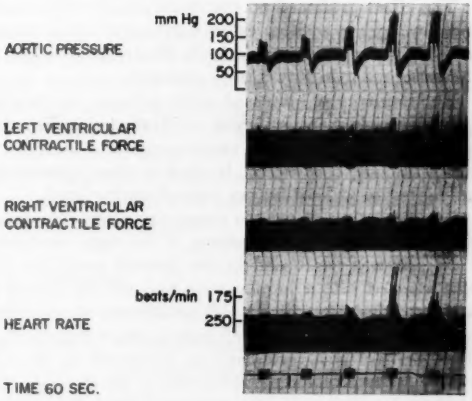

Fig. 3. Left and right ventricular contractile force and heart rate responses to a gradual increase of a mechanical aortic stricture a few millimeters distal to the origin of the left subclavian artery. Note the difference in response of left and right ventricle.

the two points of attachment of the strain gauge arch is already stretched, the length of the segment remains unaffected by moderate changes in heart size. Certain fibers, however, pass through only one point of attachment, though relatively parallel to the long axis of the arch. With enlargement of the heart these fibers would be lengthened and would accordingly contract more vigorously (COTTEN and BAY 1956), thus accounting for cases with increased strain gauge arch recording on heart enlargement. In the only experiment (cat no. 9), which involved hypothalamic stimulation in connection with left ventricular attachment of the strain gauge arch, the rise of mean aortic pressure during central stimulation was so low (15 mm Hg) that its effect on the recording of left ventricular contractile force was insignificant. This observation accords with data from similar experiments performed by COTTEN and BAY.

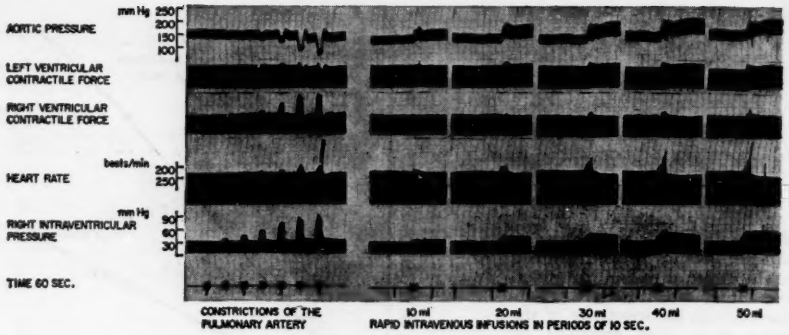

Fig. 4. Left and right ventricular contractile force, right intraventricular pressure, and heart rate responses to a gradual increase of a mechanical stricture of the pulmonary artery and to rapid intravenous blood and/or dextran infusions.
Note the slight increases of contractile force, if any, with rapid intravenous infusion. The weight of the cat was 2.7 kg.

The effect produced on the strain gauge arch recording of left ventricular contractile force by a rise in aortic pressure due to artificial stricture of the thoracic aorta, was studied in 4 experiments (one of which is illustrated in Fig. 3). Results similar to those found in the experiment in Fig. 3 were obtained.

The influence of increased aortic pressure on the strain gauge arch recording from the right ventricle is insignificant, as illustrated in Fig. 3. This fact accords with the results obtained in the other 12 experiments in which the abdominal or the thoracic aorta was artificially constricted. In each of these experiments the rise of mean aortic pressure amounted to at least 50 per cent of resting level.

Augmentation of venous return by rapid intravenous infusion of dextran influences the strain gauge arch recording of the right ventricle to only a slight degree (COTTEN 1953), as was confirmed in the present study. In 7 experiments in which a cannula was inserted into a jugular vein and the tip placed close to the entrance to the right atrium, infusion of blood and/or dextran in amounts up to 4—7 ml per second over periods of 5—10 sec, caused only a slight increase in the strain gauge arch recording. The increase is insignificant as compared to the relatively substantial augmentation caused by hypothalamic activation of the inotropic fibers to the heart. The response to gradual increase of venous return as well as to artificial constriction of the pulmonary artery in the experiment illustrated in Fig. 4, is in line with the effects obtained in the other six experiments. The cardiac output in a cat of 2.5 kg is reported to be in the region of 270 ml per minute (SPECTOR 1956). Assuming that the resting cardiac output in my experiments amounted to 250—400 ml, the venous return to the heart must have reached almost double that value in the infusion experiments. The maximal increase of venous return which may, in the relevant experiments, be elicited by the fairly mild central stimulation of the sympathetic vasodilator outflow is probably of the same magnitude as that produced by the infusions.

Because of the above considerations and for the sake of a convenient operative approach, the right ventricle was considered preferable to the left in experiments in which hypothalamic stimulation was applied in conjunction with measurement of cardiac contractile force.

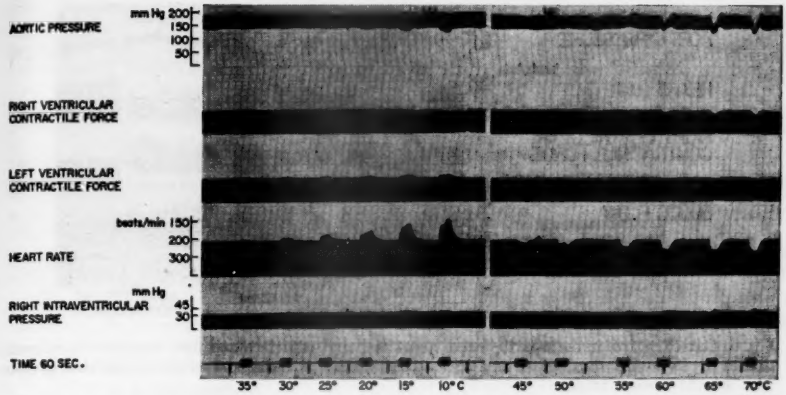

Fig. 5. Left and right ventricular contractile force and right intraventricular pressure responses to alterations of the heart rate produced by topical application of saline of varying temperature on the right atrium.
Note the minimal responses of contractile force to the varying heart rates.

That the effect of heart-rate changes on the strain gauge arch recording of contractile force is negligible (COTTEN 1953), was confirmed in 6 of my experiments. In 3 of these a pacemaker with the two electrodes separated by 3 mm was attached to the right atrium and stimulated with various frequencies (2.0 V and pulse duration 1.5 msec); in the other three, saline solution of varying temperature (Fig. 5) was topically applied to the region of the right atrium where the sinu-atrial node is situated, and produced alterations of heart rate. In each of the experiments an increase of 20 to 70 per cent in the range of 150—300 beats per minute was evoked with insignificant responses in the strain gauge arch recording of cardiac contractile force.

Results

Cardiac Responses to Hypothalamic Stimulation Producing Muscle Vasodilatation

In the 18 experiments in which skeletal muscle vasodilatation was produced by hypothalamic stimulation, cardiac contractile force and heart rate were continuously recorded. Fig. 6 illustrates a typical experiment. "Hyp I" in the figure indicates a response to stimulation in the anterior part of the hypothalamus whereby the sympathetic vasodilator outflow was activated. After a latency of 4 sec or less, muscle vasodilatation, tachycardia and an increase of right ventricular contractile force appeared. The short latency of the responses was consistent with that in all except two of the other experiments; the two exceptions will be discussed later on. Intravenous injection of atropine 0.3 mg/kg blocked the muscle vasodilator response to stimulation, a fact suggestive of a true cholinergic effect. In all experiments in which atropine was applied, the muscle vasodilator response was either markedly depressed or abolished. The cardiac responses were not affected by atropine either in the experiment illustrated in Fig. 6, or in the other experiments, but almost disappeared on removal

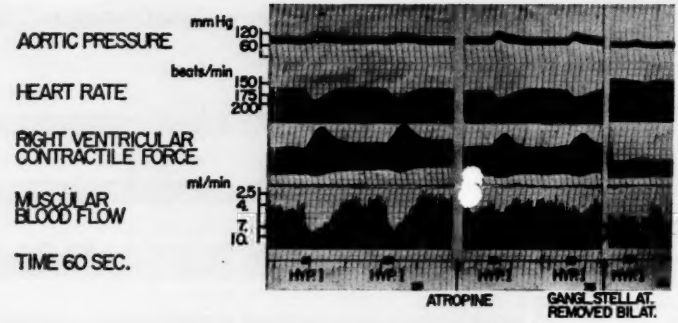

Fig. 6. Cat 17. Cardiac contractile force, heart rate and muscle blood flow responses to stimulation in the hypothalamus before and after atropinization and removal of the two stellate ganglia. The adrenals were intact.
HYP. I. Hypothalamic stimulation 2.0 V, 70 cps.
Atropine 0.3 mg/kg intravenously.
The figures indicate the elapsed time in minutes after the completion of preparation.
Note that there is no change in muscle blood flow on stimulation after atropine and that the cardiac responses almost disappear following removal of the two stellate ganglia.

of the two stellate ganglia. As will be seen in the figure, stellatectomy in itself caused a reduction of about 50 per cent in the contractile force. A similar reduction was produced in some of the other experiments, but more often the decrease was less. In 9 experiments in which right intraventricular pressure was measured, stellatectomy caused a slight reduction of the systolic pressure concomitantly with the decrease in contractile force (Rosén 1961 a).

In all experiments cardiac augmentation accompanied the muscle vasodilator response following hypothalamic stimulation (see Table I and II). The short latency of cardiac responses to hypothalamic stimulation suggests that nervous mechanisms are implicated. Evidence of this was found both in the experiment illustrated in Fig. 6 and in others (Table II) in which removal of the two stellate ganglia caused a marked depression or abolition of the cardiac responses.

In 2 experiments (cat 12 and 13 in Table II) carotid occlusion was produced in the interval between two identical hypothalamic stimulations and was maintained during the second stimulation. The magnitude of the cardiac response was the same after each stimulation, being unaffected by the persistent carotid occlusion. In one experiment (cat 6 in Table I) carotid occlusion had been done before any hypothalamic stimulation was carried out. Bilateral vagotomy was performed in seven experiments prior to hypothalamic stimulation.

In 3 experiments (cat 2, 9 and 17 in Table I and II) the intestinal blood flow was recorded during central stimulation of the sympathetic vasodilator pathway. Vasoconstriction occurred in each case, a fact which accorded with earlier observations (Eliasson *et al.* 1951).

Table I. Responses of cardiac contractile force (C. F.) and heart rate (H. R.) to hypothalamic stimulation of the sympathetic vasodilator pathway eliciting a muscle vasodilator response

Cat. no.	C. F.	H. R.
1 A	+++	++
2 A	++	not measured
3	++	0
4	+++	not measured
5	++	++
6 V, PCO	++	++
7	+++	++
8	+++	+

A = Adrenalectomized
V = Vagotomized
PCO = Persistent carotid occlusion

C.F. +++ correspond to the response of about 1.0 μg/kg adrenaline i.v.
++ correspond to the response of about 0.5 μg/kg adrenaline i.v.
+ correspond to the response of about 0.2 μg/kg adrenaline i.v.
0 insignificant response

H.R. +++ 15—20 % increase
++ 10—15 % increase
+ 5—10 % increase
0 < 5 % increase (insignificant response)

Table II. Responses of cardiac contractile force (C. F.) and heart rate (H. R.) to hypothalamic stimulation of the sympathetic vasodilator pathway (eliciting a muscle vasodilator response) following adrenalectomy and/or stellatectomy

Cat. no.	C. F.	H. R.	After adrenalectomy		After stellatectomy	
			C. F.	H. R.	C. F.	H. R.
9	++	+	+	0		
10 V.....	+++	+	0	0		
11 V, S.....	+++	0	0	0		
12 V, PCO	+++	+	+++	+	0	0
13 V, PCO	+++	++	+	+	0	0
			After stellatectomy		After adrenalectomy	
14 A.....	++	++	0	0		
15 V.....	+++	++	0	0		
16	+++	+	+	+		
17	++	+++	0	+	0	0
18 V.....	++	+++	+	++	0	+

S = Stellatectomized
A, V, PCO, C. F. (+++, ++, +, 0) and H. R. (+++, ++, +, 0) see table I.

The Magnitude of Increased Cardiac Contractile Force and Heart Rate

In all experiments the increase in cardiac contractile force following hypothalamic stimulation of the sympathetic vasodilator pathway was estimated by determining the dose of adrenaline required to produce, via intravenous injection, a like increase in the force of contraction. The adrenaline was injected not long before or after the hypothalamic stimulation to insure that the cardiac contractility conditions would not differ from those under the hypothalamic activation.

Difficulties were encountered in making a percentual calculation of the observed increases of cardiac contractile force, since the cardiac responses to dentical repeated hypothalamic stimulation sometimes varied substantially in a given experiment, particularly when the intervals between stimuli were protracted. In these experiments it was found, however, that the responses varied quantitatively in the same way as those produced by intravenous injection of adrenaline.

The increase of cardiac contractile force accompanying vasodilatation in skeletal muscle on stimulation of the sympathetic vasodilator outflow was, under the present experimental conditions, of the same magnitude as that following intravenous injection of 0.5 to 1.0 μg adrenaline per kg (see Table I and II). The heart acceleration, as will be seen from Table I and II, generally amounted to between 5 and 20 per cent.

Following removal of the stellate ganglia, and likewise after adrenalectomy, adrenaline was always injected once more for estimation of any augmentation of the contractile force with further hypothalamic stimulation. The effect of adrenaline on the contractile force was always at least as pronounced after blocking of the sympathetic cardiac innervation as it had been before (Fig. 7).

Influence of Catechol Release from the Adrenals on Cardiac Responses and on Muscle Vasomotor Responses

In 8 experiments the influence of catechol output from the adrenals on cardiac responses following hypothalamic stimulation of the sympathetic vasodilator pathway was determined (Table II). In three the original response of cardiac contractile force was partly inhibited after adrenalectomy. In a further two (cat 10 and 11) the response was abolished. The latter finding suggests that the effects were elicited entirely by released catechols — a possibility which is supported by the relatively long latency (10—12 sec) of the cardiac responses in these two experiments. The muscle vasodilator response appeared, however, within 4 sec after the onset of stimulation in these two experiments. The heart acceleration was reduced or abolished in 5 experiments by adrenalectomy.

In two or three of the five cats in which stimulation caused a catechol release from the adrenals of sufficient magnitude to influence the force of cardiac contraction, the adrenaline output may even have sufficed to produce slight muscle vasodilatation.

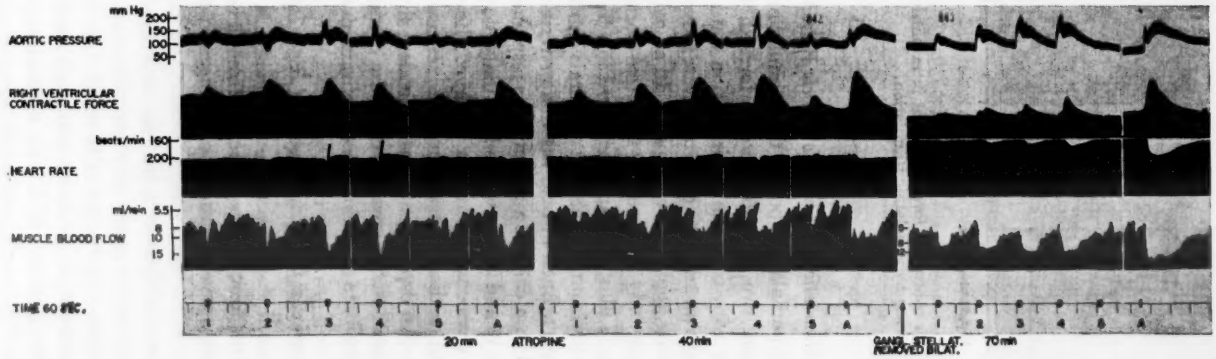

Fig. 7. Cat 16. Cardiac contractile force, heart rate and muscle blood flow responses to stimulation at various levels in the hypothalamus before and after atropinization and removal of the two stellate ganglia. The adrenals were intact.

1—5: Hypothalamic stimulation (2.0 V, 70 cps) delivered at five horizontal levels approximately 1 mm apart while the electrode was gradually shifted ventrally.

A. Adrenaline 1.0 μg/kg intravenously.

Atropine 0.5 mg/kg intravenously.

The figures in minutes indicate the elapsed time after the completion of preparation.

Note that the muscle vasodilator responses appear concomitantly with an increase of cardiac contractile force during ventral shifting of the electrode.

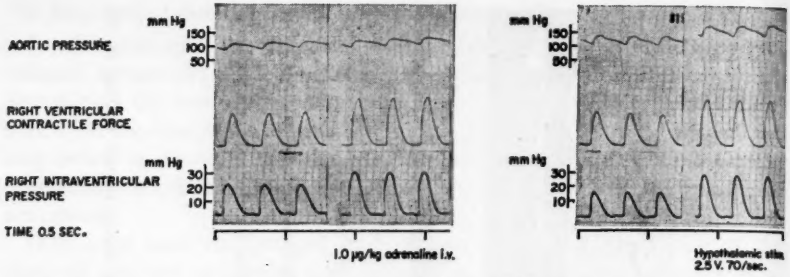

Fig. 8. Cat 19. Tracings of right ventricular contractile force and right intraventricular pressure following injection of adrenaline and during hypothalamic stimulation.

Cardiac Responses to Hypothalamic Stimulation Eliciting No Muscle Vasodilatation

During attempts to locate hypothalamic points the stimulation of which would elicit muscle vasodilatation, two methods were performed. In one, the electrodes were moved ventrally with continuous stimulation until a response was obtained. In the other (8 experiments), the electrodes were moved ventrally by one mm steps, stimulation being applied at each point. In all experiments a muscle vasodilator response apparently occurred at the same horizontal level as did cardiac augmentation. Fig. 7 illustrates an experiment in which identical stimuli were delivered at various horizontal levels, the numerals 1 to 5 indicating levels approximately one mm apart. Each muscle vasodilator response elicited by stimulation was accompanied by a cardiac augmentative response.

In 2 of the 20 experiments (cat 19 and 20) in which hypothalamic stimulation was applied, moderate muscle vasoconstriction was observed, however, concomitantly with increased force of cardiac contraction. The increase of contractile force and heart rate in these two experiments was equivalent to that in the experiments in which muscle vasodilatation accompanied the cardiac response and appeared, in addition, within 4 sec after the onset of stimulation.

Simultaneous Recording of Right Ventricular Contractile Force and Right Intraventricular Pressure

Alterations in the duration of the isometric pressure gradient as well as other changes of the pulse contour may serve as criteria of changes in the force of ventricular contraction.

Right intraventricular pressure was measured, in four experiments (cat 3, 10, 12 and 19), concurrently with strain gauge arch recording of contractile force from the right ventricle. One experiment is illustrated in Fig. 8. The pulse contour of the intraventricular pressure was compared with the strain gauge arch recording after injection of 1.0 μg adrenaline per kg and during hypothalamic stimulation. The tracing was made a few seconds after the

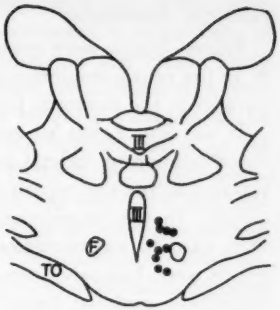

Fig. 9. Drawing of a frontal section through a cat's hypothalamus 3—4 mm posterior to the anterior commissure.

Stimulated points, histologically localized to a region 1.5 mm anterior or posterior to the section, are represented by the black and the dotted white circles. All points were associated with increased cardiac contractile force when stimulated.

(F, fornix; TO, optic tract; III, third ventricle.)

● Cardiac augmentation and muscle vasodilatation

⊙ Cardiac augmentation and muscle vasoconstriction

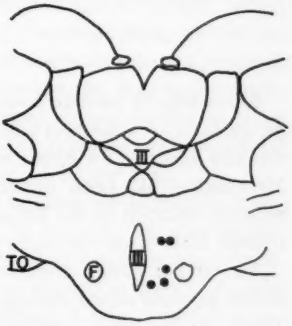

Fig. 10. Drawing of a frontal section through a cat's hypothalamus 7—8 mm posterior to the anterior commissure. Stimulated points, histologically localized to a region 1.5 mm anterior or posterior to the section, are represented by the black circles. All points were associated with increased cardiac contractile force and muscle vasodilatation when stimulated.

(F, TO and III, see text to Fig. 9.)

onset of increase in amplitude of the strain gauge arch recording. Both adrenaline and hypothalamic stimulation increased the contractile force as measured by the strain gauge arch. Concomitantly the pulse contour revealed a diminution in duration of the isometric pressure gradient, an accelerated rise in tension, increased systolic pressure, and more rapid relaxation. In the other 3 experiments, intraventricular pressure responses of the same character were obtained. In none of the 4 experiments was the rise in heart rate sufficient to account for the decreased duration of the isometric pressure gradient. A change of diastolic pressure in the pulmonary artery causing the decreased duration response probably did not occur. The observed changes of pressure conform to parameters which other authors consider to be indicative of increased force of cardiac contraction during stimulation of the stellate ganglia (Anzola and Rushmer 1956, Kelso and Randall 1959). The present results are in agreement with those found by Cotten and Bay (1956) in their investigation of the action of cardioactive drugs on the strain gauge arch recording and on the duration of the isometric pressure gradient.

Histologic Examination

Fifteen brains were examined histologically. After removal they were fixed in 10 per cent formaldehyde solution. Frozen serial sections were cut in a plane parallel to the electrode position.

It was found that all localized points the stimulation of which had elicited vasodilatation in skeletal muscle and concomitant cardiac augmentation, were situated within the hypothalamic vasodilator region reported by LINDGREN *et al.* (1956). In two experiments muscle vasoconstriction occurred concomitantly with cardiac augmentation upon hypothalamic stimulation. The stimulated points were, however, found to be situated within the region through which the vasodilator pathway passes (Fig. 9). In Fig. 9 and 10, sections of the hypothalamus show the 13 localized points which, when stimulated, were associated with muscle vasodilatation and increased contractile force.

Discussion

Recording of cardiac contractile force is readily accomplished with a strain gauge arch — a method which Cotten and coworkers have shown to be exceptionally sensitive for direct measurement of acute changes in the contractility. Its validity has been further substantiated in the present experiments. For reasons reported in the foregoing, it was found appropriate to record the contractile force from the right ventricular muscle.

The strain gauge arch technique is highly serviceable for experiments in which no substantial changes occur in the size of the heart (COTTEN and BAY 1956), as has been confirmed in the present investigation. Since the muscle segment between the two points of attachment of the strain gauge arch is stretched, the tracing is not affected to any noticeable extent by the occurrence of a moderate heart enlargement, though it may be appreciably influenced by a more pronounced heart enlargement. If hypothalamic stimulation produced an enlargement of the right ventricle, the change in ventricular size was probably insufficient to influence noticeably the strain gauge arch recording. This was demonstrated by studying the degree to which factors determining the heart volume influenced the strain gauge arch recording from the right ventricle. The tracing was not noticeably affected by an increase either in aortic pressure or in the venous return to the heart, of magnitudes such as could result from the relevant hypothalamic stimulation. Cardiac acceleration does not significantly influence the strain gauge arch recording (COTTEN 1953), as was confirmed in my investigations.

Recent studies have demonstrated the presence, in the sympathetic outflow, of fibers which affect the contractile force of the heart (COTTEN 1953, ANZOLA and RUSHMER 1956, RANDALL and ROHSE 1956). Stimulation of these fibers increases the contractile force without necessarily raising the heart rate (RANDALL and ROHSE 1956). The central representation of these inotropic fibers to

the heart is obscure, though RUSHMER *et al.* (1959) and SMITH *et al.* (1960) have demonstrated that the left ventricular stroke work is increased by stimulation of a few circumscribed areas in the hypothalamus. MANNING and PEISS (1960) have drawn, solely on the basis of blood pressure changes, conclusions regarding augmentation of the cardiac contractile force. They observed increases of the "contractile force" on stimulating large areas of the hypothalamus. — As regards the central representation of inotropic fibers to the heart, my investigations have confirmed the Rushmer group's observations and have also demonstrated the presence of a single, relatively well-circumscribed area, extending from the anterior to the posterior hypothalamus, the stimulation of which increases the cardiac contractile force. Under the relevant experimental conditions the augmentation was equivalent to that produced by intravenous injection of adrenaline 0.5—1.0 μg/kg.

It has long been known that various hypothalamic structures can, when activated, influence the heart rate. RUSHMER and his co-workers, as well as MANNING and PEISS, frequently observed elevation of the heart rate as a result of hypothalamic stimulation. It was found in my investigation that tachycardia generally accompanied the increase of contractile force elicited by the relevant stimulation in the hypothalamus. Under the prevailing experimental conditions the increase in heart rate amounted to a maximum of 20 per cent.

Those areas the stimulation of which produced an increase of cardiac contractile force and usually tachycardia, appeared to coincide in localization with the sympathetic vasodilator outflow, which passes through the hypothalamus (LINDGREN *et al.* 1956). When vasodilatation was elicited in skeletal muscle by stimulation of the sympathetic vasodilator pathway, it was invariably accompanied by cardiac augmentation. However, in 2 of the 20 experiments in which hypothalamic stimulation was applied, a moderate muscle vasoconstrictor response appeared concomitantly with an increased cardiac contractile force. The histologic localization of the two stimulation points coincided with other stimulation points located within the area through which the sympathetic vasodilator pathway passes. It is possible therefore, that in these experiments skeletal muscle vasodilatation was concealed by vasoconstriction. ELIASSON *et al.* (1951) observed that vasoconstrictor effects could occur in the muscle simultaneously with vasodilatation, on central stimulation of the vasodilator pathway.

In general the cardiac effects were either partly or entirely mediated by the sympathetic nerves, since in almost all experiments (18 out of 20) the latency was 4 sec or less. Also removal of the stellate ganglia in those animals with the short latency reduced or abolished the responses. The role of the adrenal medulla in the cardiac responses varied. In two experiments adrenalectomy abolished the cardiac responses; in others there was either partial reduction or no effect. GRANT *et al.* (1958) reported that under their experimental conditions, one adrenal gland releases on the average 0.32 μg/kg/min adrenaline and 0.35

μg/kg/min noradrenaline upon hypothalamic stimulation in the sympathetic vasodilator area. Intravenous adrenaline or noradrenaline infusion of 0.1—0.5 μg/kg/min is considered to be sufficient to cause an increase of cardiac contractile force (ROSÉN 1961 b). Since the present experimental conditions are similar to those of GRANT *et al.* and of ROSÉN it is not surprising to find that cardiac augmentation partly due to catechols released from the adrenals, occurs when the vasodilator outflow is activated in the hypothalamus.

The present experiments showed that cardiac augmentation probably resulting in an increased output, as well as skeletal muscle vasodilatation, splanchnic vasoconstriction, and catechol discharge from the adrenals were characteristic responses to hypothalamic stimulation of the vasodilator system. The cardioactive fibers found in this investigation were associated with the "vasodilator area", since it was not possible to demonstrate them immediately outside it. The question arises whether the concurrence of these responses is purely coincidental or whether it points to a functional entity. To judge from the results, the hypothesis of a functional entity is plausible. Such an assumption is further supported by the observation that an increased force of cardiac contraction as well as tachycardia occur on stimulation of the vasodilator pathway in the oblongate medulla (ROSÉN 1961 a).

There are several conditions associated with a cardiovascular pattern of the same type as that obtained by hypothalamic activation of the sympathetic vasodilator system. One such condition is emotional excitement. Increased cardiac output (STEAD *et al.* 1945, HICKAM, CARGILL and GOLDEN 1948), vasodilatation in skeletal muscle (BLAIR *et al.* 1959), and increased catechol release, mainly of adrenaline, from the adrenals (for references see EULER 1956), are all relevant to emotional stress. Furthermore, in this condition the hypothalamus is known to play an important integrative role. That the sympathetic vasodilator pathway may be activated during conditions of emergency was suggested by ABRAHAMS and HILTON (1958). They stimulated the hypothalamic vasodilator pathway in anesthetized cats and allowed the animals to recover consciousness with the electrode *in situ*. Repeated stimulation produced responses described as "flight and fight reactions". In another series of experiments on conscious cats ABRAHAMS *et al.* (1960a) observed, following acoustic and nociceptive stimulation, a rise of temperature in the venous blood from the skeletal muscle. They suggested that this response might be caused by muscle vasodilatation elicited by activation of the sympathetic vasodilator outflow. The vasodilator response was presumed to be part of a defence reaction.

The fact that impulses from the brain may produce cardiac augmentation, cardiac acceleration and muscle vasodilatation also leads to other speculations. RUSHMER *et al.* (1959) pointed out that the presence of such mechanisms appears essential to an explanation of the very abrupt responses at the onset of exercise and of the cardiovascular adjustments occurring in anticipation of exercise. They found that changes of cardiac functions following hypothalamic stimula-

tion were distinctly similar to treadmill exercise responses. The fact that the sympathetic vasodilator outflow has a cortical origin (ELIASSON, LINDGREN and UVNÄS 1952) is one of the reasons for the inference that it may be involved in the initial phase of muscle exercise. The present finding that augmented cardiac contraction, tachycardia, and muscle vasodilatation occur concomitantly in response to hypothalamic stimulation supports the hypothesis that the vasodilator system may be concerned with the initial adjustment of the muscular blood flow during exercise.

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Further Observations on Anaphylactic Mast-Cell Changes in the Nasal Mucosa of Guinea Pigs in Vivo

By

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Abstract

BORÉUS, L. O. *Further observations on anaphylactic mast-cell changes in the nasal mucosa of guinea pigs in vivo.* Acta physiol. scand. 1961. *52.* 309—319. — The anaphylactic reactions of tissue mast cells were quantitatively studied in the nasal mucosa of anaesthetized guinea pigs *in vivo.* Intra-arterial challenge with antigen produced extensive disappearance of the mast cells, as well as anaphylactic shock symptoms, in animals sensitized passively with rabbit anti-ovalbumin or actively by intranasal application of ovalbumin. ACTH and cortisone in high doses did not prevent the development of active anaphylaxis, nor did they significantly alter the average mast-cell population of the tissue. Moreover, the cell reaction to antigen was not antagonized by pretreatment with these hormones. Heating of the animal prior to challenge, and prolongation of the injection time diminished the anaphylactic mast-cell reaction and the intensity of shock symptoms. The "missing" mast cells were seen to reappear in about 7 weeks. On administration of antigen *in vivo* they again displayed a strong disappearance reaction.

Administration of antigen to the nasal mucosa of sensitized guinea pigs *in vivo* causes a rapid and extensive decrease in the mast-cell count, which may be studied quantitatively (BORÉUS 1960). In this investigation the method was used to study the local anaphylactic mast-cell response under various experimental conditions; namely, after passive sensitization of the animal with rabbit antiserum and after active sensitization by topical, intranasal application of egg albumin. In addition, the possible influence of ACTH and cortisone on the development of active anaphylaxis has been studied. Finally, observations relating to inhibition of the anaphylactic mast-cell disappearance and to the reappearance of previously abolished mast cells in the mucosa are reported.

Methods

Guinea pigs of both sexes were used.

Active sensitization was produced, unless otherwise stated, by one subcutaneous injection and, three days later, one intraperitoneal injection of 100 mg crystalline egg albumin. Intra-arterial challenge was done in the anaesthetized animal via the common carotid artery with 1 ml of a 10 % w/v solution of egg albumin in saline. The injection time was 1 minute. Two specimens of nasal mucosa were removed from one side prior to the injection of antigen, and two specimens were taken from the contralateral side 5 minutes after the injection. The mast-cell populations on the two sides were estimated and compared. Anaphylactic "disappearance" of mast cells was expressed as a percentual decrease in relation to the control. The details of this method have been previously presented (BORÉUS 1960).

The anti-ovalbumin rabbit serum used for passive sensitization of the guinea pigs was obtained by heart puncture of a rabbit which had received several intravenous and intraperitoneal injections of egg albumin during the preceding 3 weeks. The same serum was used in all experiments. The strength of the antiserum was roughly estimated by means of a quantitative precipitation test, which disclosed a titer of 1 : 10,000.

Results

I. *Development of anaphylactic mast-cell sensitivity*

Passive sensitization. Previous work on guinea-pig mast cells *in vivo* (BORÉUS 1960, 1960 a) had been done with actively sensitized guinea pigs. A few experiments were now performed to ascertain if a similar mast-cell reaction *in vivo* could be induced following passive sensitization.

Three animals were given 1, 2 and 4 intraperitoneal injections, respectively, of 1 ml rabbit anti-ovalbumin serum. The injections were given on consecutive days, and the animals were challenged on the day after the last injection. The resulting mast-cell disappearance was for 1 ml, 62 and 79 %; for 2 ml, 86 and 95 %; and for 4 ml, 86 and 95 %. It was noted that the anaphylactic shock symptoms were fairly mild in all animals despite the intensive mast-cell reaction. An increased respiratory rate was observed in one animal, and urination and defaecation in another, but no signs of severe broncho-constriction appeared and no anaphylactic death occurred.

To test the duration of the passively induced anaphylactic state of the mast cells, 5 other normal guinea pigs were given 2 injections, each of 1 ml rabbit anti-ovalbumin serum, on two subsequent days. They were challenged with egg albumin 6, 11, 12 and 13 days after the first injection. The fifth animal was tested on day 15 with 1 ml rabbit serum intra-arterially. The mast-cell response in these 5 animals is shown in Fig. 1. The animal challenged at day 6 was the only one to suffer fatal shock; the others showed no symptoms. The last animal tested with rabbit serum on day 15 reacted with intensive, fatal anaphylactic shock, indicating that it had developed active anaphylactic sensitivity to the serum. Control experiments had shown that similar serum injections in normal animals did not affect the mast cells.

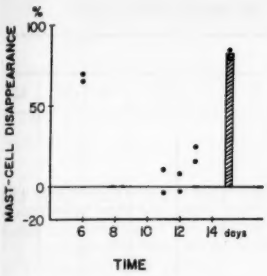

Fig. 1. Anaphylactic mast-cell disappearance from nasal mucosa of guinea pigs at various intervals after passive sensitization with rabbit anti-ovalbumin serum. Challenge dose 100 mg egg albumin. Two values for each animal are given. The hatched column denotes the mean of two disappearance values obtained in one animal upon challenge with 1 ml rabbit serum on day 15.

Active intranasal sensitization. Four normal guinea pigs were anaesthetized and placed in "hanging head" position. By means of a thin, slightly bent steel cannula, 4 portions of 0.02 ml (2 mg) egg albumin were deposited, with 15-minute intervals, on the inferior nasal concha, the cannula being introduced through the left nostril. Great care was taken not to injure the mucosa. Except for some sneezing, the application produced no symptoms. In this way, each animal received 8 mg egg albumin intranasally. One, 3, 5 and 7 days later, the whole procedure was repeated, so that each animal received, in all, 40 mg egg albumin via the left nasal mucosa. (It should be pointed out, however, that a part of that amount had been ejected from the nasal cavity by sneezing.) About 4 weeks later, 3 of the animals were anaesthetized and challenged with antigen intravenously, intra-arterially (left side) and intra-arterially (right side), respectively. The fourth animal, however, was sacrificed by a blow on the head and the nasal mucosa subjected to gross and microscopic examination. No injury of the mucosa was detectable and the mast cells on both sides appeared quite normal. The result of the challenge was as follows: intravenous injection: relatively severe anaphylactic shock with strong inspiratory movements, cyanosis and urination, accompanied by almost total disappearance of mast cells, on both the left and the right sides; intra-arterial injection: lethal shock with disappearance values 91 and 90 % (left side) and 99 and 96 % (right side).

In a later series of 6 guinea pigs a much smaller intranasal dose of egg albumin was shown to be sufficient to induce local and general anaphylactic sensitivity. Two of the 6 animals were treated on days 1, 2 and 4 as described above, each time with 8 mg egg albumin intranasally. Two animals were treated on days 1 and 2, and two were treated once only, on day 1. When challenged 3 weeks later with egg albumin the twice or thrice treated animals succumbed to anaphylactic shock. The values for mast-cell disappearance in these animals ranged between 80 and 93 %. Of the two animals which received only a single treatment, one showed no shock symptoms when challenged, whereas the other evinced comparatively severe shock. The mean mast-cell disappearance was 43 %.

Table I. Dose schedule for ACTH, cortisone and antigen during the sensitization period

	Day											
	1	2	3	4	5	6	7	8	9	10	11	12
Group 1												
Antigen ..... mg	100			100								
ACTH ..... IE	10	5	5	5	2.5	2.5	2.5	2.5	2.5	1.25	1.25	1.25
Group 2												
Antigen ..... mg	100			100								
Cortisone acetate .. mg	25	12.5	12.5	12.5	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25

Influence of ACTH and cortisone on the development of active sensitization. Two groups of 5 guinea pigs each were sensitized in the usual way (one subcutaneous and, three days later, one intraperitoneal injection of 100 mg egg albumin), those in the first group being simultaneously treated with ACTH and those in the second with cortisone. The dose schedule is given in Table I.

On the thirteenth day the animals were challenged with an intra-arterial injection of 100 mg egg albumin. All of them reacted with fatal anaphylactic shock. The mean values for mast-cell disappearance were in the ACTH-treated group 78.4 $\pm$ 4.8 %, and in the cortisone-treated group 84.6 $\pm$ 2.8 %. These values did not differ significantly from the corresponding mean value for untreated, actively sensitized guinea pigs, challenged with like doses of antigen (cf. BORÉUS 1960). That treatment with these two hormones did not significantly reduce the mast-cell population of the nasal mucosa was established by comparing the values for the hormone-treated animals with 6 values for non-treated guinea pigs of the same stock. The values were: ACTH-treated 3.2 $\pm$ 0.6, cortisone-treated 3.0 $\pm$ 0.3, and untreated controls 3.0 $\pm$ 0.6 cells per 0.256 mm^2.

II. *Modification of the mast-cell response to antigen*

Pretreatment with ACTH and cortisone. Two actively sensitized guinea pigs received 10 IE ACTH subcutaneously and two received 25 mg cortisone acetate subcutaneously. About 3 hours later they were challenged with an intra-arterial injection of 100 mg egg albumin. All four animals evinced fatal anaphylactic shock and an intense mast-cell reaction. The disappearance was (mean of 4 values each): in the ACTH-group 91.0 $\pm$ 2.3 % and in the cortisone-group 86.3 $\pm$ 9.8 %.

Heating of the animal. The effect of elevated temperature on the mast-cell sensitivity to antigen was tested by heating the anaesthetized, tracheotomized guinea pig in a thermostatically regulated chamber (at 50—55° C) until the

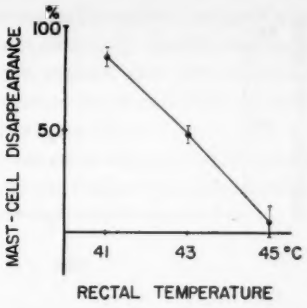

Fig. 2. Inhibition of anaphylactic mast-cell disappearance from the nasal mucosa of guinea pigs following heating of the animal. Challenge dose 100 mg egg albumin. Means and standard errors of 6 disappearance values.

desired rectal temperature had been attained (41—45° C). At that point, (reached after 30—40 minutes), the animal was removed from the box and the rectal temperature was allowed to fall to 38—39° C. The animal was then challenged intra-arterially in the usual way. The inhibitory effect of heating is evident from Fig. 2. The heating procedure was usually accompanied by some degree of respiratory and circulatory depression, though anaphylactic symptoms were still discernible in all three of the animals heated to 41° C as well as in one of those heated to 43° C. No shock symptoms could be detected in the other animals.

Variation of injection time. Twenty-five mg of antigen was administered intra-arterially to sensitized guinea pigs. The injection volume was in each instance 1 ml, but the injection time varied: in three animals it was 1 minute; in four, 10 minutes; and in three, 30 minutes. A constant injection rate was assured by use of an infusion pump.

Preliminary experiments had exposed one drawback to this type of study, i. e. the fact that some animals developed severe, often fatal anaphylactic shock within the 10-minute or 30-minute period. Thus, as a precaution, all animals in this series were pretreated with 1.25 mg promethazine, administered intraperitoneally about one half hour before challenge. Previous experiments had shown that this antihistamine treatment did not significantly influence the anaphylactic mast-cell disappearance in nasal mucosa but sufficed to protect the animals from symptoms of anaphylactic shock.

The disappearance values were as follows: 1 minute, 92.0 ± 2.7 %; 10 minutes, 78.8 ± 4.5 %; and 30 minutes, 22.7 ± 13.9 %. Thus, the standard error is higher in the 30-minute group than in the others. Since these animals were pretreated with an antihistaminic drug which precluded anaphylactic symptoms, no comparison of the latter was possible. Statistical comparison of the disappearance values for the three groups gave the following result: 30-minute group compared to 1-minute group, $P < 0.001$; 30-minute group compared to 10-minute group, $0.001 < P < 0.01$; 10-minute group compared to 1-minute group, $0.01 < P < 0.05$.

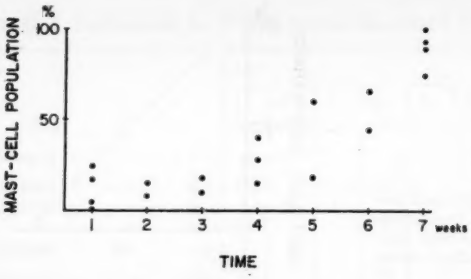

Fig. 3. Reappearance of mast-cells in the nasal mucosa of guinea pigs at varying intervals after anaphylactic disappearance. Mast-cell population expressed in percent of the population on the untreated control side.

III. *Reappearance of mast cells following anaphylactic disappearance*

Local application of antigen upon the nasal mucosa of sensitized guinea pigs produces a rapid and extensive disappearance of mast cells (Boréus 1960). The purpose of the present experiment was to determine the time required for reappearance of the cells. The above-described procedure for intranasal administration of antigen was employed, 5 portions of 2 mg antigen each being dissolved in 0.02 ml saline and introduced into the left nasal cavity. These volumes were too small to be aspirated and thus lead to anaphylactic shock.

Following this treatment the animal had, on the right side, an intact nasal mucosa and on the left a mucosa which had undergone a local anaphylactic reaction with mast-cell depletion. The animals were killed after varying intervals and the difference in mast-cell count between the right and left sides was estimated. The results of these experiments are shown in Fig. 3. The application of antigen resulted in a sharp decline in mast-cell population. Total disappearance of the mast cells was at no time observed, however. After about 3 weeks the mast cells began to reappear. They were smaller than normal and the metachromatic material was sparse and faintly stained. Within approximately 7 weeks, most of the mast cells had reappeared and had regained their usual size and staining characteristics.

Attempts were made to maintain the low mast-cell population following local anaphylaxis. In several animals, therefore, antigen was reapplied intranasally 5 weeks after the first treatment. When the animals were killed a few weeks later, however, the left nasal cavity, and in some cases the right one, was found to be severely infected and suppurative. The inferior nasal concha was entirely or partly necrotic and in some animals no concha whatsoever, remained. Histological examination of the remnants of the mucosa revealed, in some instances, a tremendous accumulation of mast cells distributed throughout the concha.

Some of the animals were challenged with antigen following removal of the specimens. They all developed severe shock, showing that the intranasal administration of antigen had not been sufficient to produce appreciable desensitization (*cf.* Boréus 1960 a).

Two animals treated 8 weeks earlier with intranasally applied antigen were challenged with the standard dose of antigen intra-arterially. Before challenge, one specimen was taken from each side to make certain that the mast cells had returned in the usual way. The antigen injection produced fatal shock in both animals, and gave rise to a strong mast-cell reaction: 77.3 $\pm$ 3.2 %. This experiment indicates that the mast cells which have reappeared following anaphylactic reaction, disappear at approximately the former rate upon further antigen administration.

Discussion

The causal relationship between formation of antibodies and development of active anaphylaxis was early confirmed by demonstration of the passive anaphylactic state in which a normal animal receives antibody formed by another animal. An essential factor in passive anaphylaxis in the guinea pig is the interval between the two injections of antibody and antigen. This latent period might reflect the time required for the antibody to become fixed on or in the cells. Such a hypothesis is supported by the finding of BENACERRAF and KABAT (1949), and that of OVARY and BIER (1953), that the latent period can be reduced with increasing doses of sensitizing antiserum.

Though cellular fixation of antibody may take place more or less uniformly in the body, it seems that, quantitatively, only a few types of cells play a major role in anaphylaxis. In the guinea pig, the destruction of tissue mast cells appears to be a principal factor in the genesis of anaphylactic symptoms. Species differences, however, are important. For instance, rabbit platelets, which contain serotonin as well as most of the blood histamine, are disintegrated in the blood by antigen *in vitro* (HUMPHREY and JAQUES 1955) and *in vivo* (WAALKES *et al.* 1957). It is probable that these cells figure predominantly in rabbit anaphylaxis. This may explain the early observation that no latent period is required for passive sensitization in this species, when both antibody and antigen are administered intravenously (DRAGSTEDT *et al.* 1940; DRAGSTEDT 1941; ROCHA E SILVA 1942). It is unlikely that platelets are of major importance in other species such as guinea pig, rat and man, since these species do not produce the high levels of circulating antibodies demonstrable in rabbit (WAKSMAN 1959). Moreover, it was shown by HUMPHREY (1955) that anaphylactic shock can be produced in guinea pigs completely deprived of circulating platelets by the use of platelet antiserum.

HUMPHREY and MOTA (1959) incubated mesentery from passively sensitized guinea pigs with antigen and demonstrated a diminution of mast-cell numbers. The present investigation shows heavy mast-cell depletion in intact tissue on antigen administration *in vivo* to a passively sensitized guinea pig. The finding that the mast-cell reaction may be elicited on the sixth but is no longer elicitable on the eleventh day after injection of the antiserum is in accord with early

observations on the duration of the passive anaphylactic state (WEIL 1917, COCA and KOSAKAI 1920). The antibody-containing serum was not only responsible for the passive anaphylaxis but initiated, at the same time, active antibody production which was demonstrable 15 days later when the mast cells showed marked disintegration in response to injection of the rabbit serum.

Local anaphylaxis without general anaphylactic shock, but manifested as disappearance of tissue mast cells, was observed in the nasal mucosa on topic application of antigen solution thereto (BORÉUS 1960). The reaction was rapid and uniform throughout the nasal concha, indicating that the antigen was easily absorbed through that tissue. This is corroborated by the finding in this study that active sensitization of the whole animal is readily induced via the intranasal route and is demonstrable both as anaphylactic shock and as a mast-cell reaction to antigen. STRÖMME (1952) reported that local application of rye and birch pollen to the nares of guinea pigs could produce active sensitization, manifested as a nasal discharge and as sneezing and scratching of the nose when the animals were challenged by pollen applied to the nares. Intravenous injection of pollen extract produced anaphylactic shock symptoms but caused no death. In this investigation an attempt was made to duplicate these experiments with the mast-cell method. Rye and birch pollen was given parenterally in large doses to normal guinea pigs. Challenge a few weeks later by means of intra-arterial or topic administration of pollen extracts, however, gave rise to neither mast-cell reaction nor anaphylactic shock symptoms.

Much work has been devoted to inhibition of anaphylactic reactions. For this type of study, *in vitro* methods such as measurement of histamine liberation from tissues, of mast-cell reaction, and of smooth-muscle contraction have been widely used since they permit numerous experiments on one animal. Studies *in vivo* have been based upon estimation of the intensity of anaphylactic shock symptoms. This intensity is difficult to evaluate quantitatively, and subjective error is not easily avoided. In an earlier paper (BORÉUS 1960 a), inhibition of anaphylactic mast-cell reaction was studied quantitatively during desensitization processes *in vivo*. The same method is here used to study the effects of ACTH and cortisone, of heating, and of slow antigen administration upon the mast-cell sensitivity to antigen.

Despite extensive study of ACTH and cortisone, the pharmacodynamics of these hormones in allergy remain obscure. Their influence on anaphylactic reactions is controversial. Although neither dose-response nor time curves were plotted in this study, the negative results with ACTH and cortisone respecting anaphylactic mast-cell response in actively sensitized guinea pig tend to substantiate the view held by most authors engaged in guinea-pig studies with various techniques (LEGER, LEITH and ROSE 1948, FRIEDLAENDER and FRIEDLAENDER 1950, MALKIEL 1951). In the present experiments the dosages of cortisone and of ACTH (which has no direct action of its own) were fairly high, and any definite inhibitory action of those drugs would have lowered the disap-

pearance values. The dose of antigen was the same as that used earlier in studies of inhibition resulting from desensitization (BORÉUS 1960 a).

The effect of the adrencoortical hormones upon formation of anaphylactic antibody is likewise unclear. Most studies of this problem seem to have been done in rabbits (for references, see ROSE 1959). That ACTH and cortisone may depress antibody levels in this species is generally agreed. GERMUTH, OTTINGER and OYAMA (1952) reported similar suppression in the guinea pig. In the present experiment high doses of both hormones failed to depress either mast-cell reaction to antigen or the anaphylactic shock. It is possible that even a meager formation of antibody suffices to sensitize the tissue mast cells (and probably other cells as well), whereby a full anaphylactic response may still occur.

ASBOE-HANSEN (1952) reported that during administration of cortisone to humans, rabbits, mice and guinea pigs, the number of mast cells in the connective tissue diminishes and vacuolization as well as alteration in the shape and outlines of mast cells take place. Similar effects were noted in the hamster by FULTON and MAYNARD (1953) and in the rat by CAVALLERO and BRACCINI (1951). On the other hand, DEVITT, PIROZYNSKI and SAMUELS (1953) found that cortisone produced no change in mast-cell frequency in various rat tissues in experiments involving numerical cell counts. ACTH and cortisone moreover, had no effect on the total number of mast cells in rat tissues (SMITH and LEWIS 1954) or in the cheek pouch of hamsters (SMITH and LEWIS 1955). The present results accord with these findings. No decrease in mast-cell frequency was detected in the nasal mucosa of guinea pigs during treatment with ACTH and cortisone, nor was a specific change in the appearance of the cells observed.

The thermal inhibition of anaphylaxis has been adduced by several authors as evidence of an enzymatic stage in this reaction. *In vivo* experiments on guinea pigs were performed by MONGAR and SCHILD (1956) who noticed that anaphylactic shock was inhibited in animals which had been heated before challenge. The present study suggests that this phenomenon may stem from inhibition of the mast-cell reaction to antigen.

Prolongation of the injection time diminished the mast-cell response, although the 30-minute values showed a high degree of variation. A possible explanation is that the tissue antibodies were partly saturated with antigen and the cells thus desensitized. This kind of anaphylactic inhibition has long been recognized (FRIEDBERGER and MITA 1912, LEWIS 1919).

Sensitized guinea pigs treated by application of antigen to the nasal mucosa exhibited almost total mast-cell depletion of the mucosa on the treated side but a normal mucosa on the untreated side. The procedure may be utilized for study of mucosal mast-cell function. In this investigation the rate of reappearance of mast cells following anaphylaxis was investigated. It appears that no such study on the guinea pig has been previously reported. The increase in

mast-cell population was found to be rather slow and of the same order as the recovery of histamine in the skin of dog and rat following injection of compound 48/80 (FELDBERG and TALESNIK 1953). It was also found comparable to the restoration rate of histamine and mast cells in the subcutaneous connective tissue and the ear skin of the rat after injection of the same drug (RILEY and WEST 1955). The reappearing mast cells were found to react promptly to readministered antigen. New-formed cells thus adopt the induced anaphylactic sensitivity pattern of the individual.

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Quantitative Differences Between Guinea-Pig Ileum and Uterus in the Schultz-Dale Reaction

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Abstract

BORÉUS, L. O. *Quantitative differences between guinea-pig ileum and uterus in the Schultz-Dale reaction.* Acta physiol. scand. 1961. *52.* 320—327. — Although the anaphylactic contraction of smooth muscle *in vitro* (the SCHULTZ-DALE reaction) is of the same type, intensity and duration in guinea-pig ileum and uterus, some quantitative differences between these two organs are demonstrable. To produce equivalent contractions by addition of histamine to the bath, a ten times higher dose was required for uterus than for ileum. In the case of uterus, this amount was more than three times the total histamine content. Uterus released much greater amounts of smooth-muscle stimulating substance during anaphylactic contraction than did ileum. In terms of histamine, 9 and less than 0.4 %, respectively, of the total content was released. Moreover, the mast-cell disappearance, demonstrable only with the highest antigen doses used, was more pronounced in uterus.

A characteristic feature of anaphylaxis in the guinea pig is smooth-muscle spasm. The pronounced contraction of the bronchiolar muscles, as well as the discharge of urine and faeces, are well-known phenomena in the anaphylactic shock syndrome. SCHULTZ (1910) and DALE (1913) demonstrated the antigen-induced contraction *in vitro* of the isolated ileum or uterus (the "SCHULTZ-DALE reaction"). Similar contraction of isolated guinea-pig tissues has been demonstrated in strips of bronchus and pulmonary artery (GROVE 1932).

The discovery of histamine in the mast cells (RILEY and WEST 1953) has given possibilities of new experimental approaches to anaphylactic phenomena. Incubation experiments with sensitized guinea-pig tissues (BORÉUS and

CHAKRAVARTY 1960) showed that anaphylactic disappearance of mast cells was correlated to the appearance of both histamine and another smooth-muscle stimulating principle, the "slow reacting substance" (SRS).

In the experiments reported here, the mast-cell reactions occurring in isolated ileum and uterus of sensitized guinea pigs following the SCHULTZ-DALE reaction were compared. By means of a non-sensitized strip of guinea-pig ileum suspended in the same bath, the amount of released smooth-muscle stimulating substance was measured simultaneously.

Methods

Female virginal guinea pigs, weighing about 300 g, were sensitized with one subcutaneous and, 3 days later, one intraperitoneal injection of 100 mg crystalline egg albumin. After an interval of 3—5 weeks the animals were sacrificed by a blow on the head and the ileum or uterus was removed, washed and immersed in Tyrode solution at room temperature pending use.

Segments of ileum or uterus, about 1.5 cm long, were suspended in a 5 ml aerated bath at 37° C. The fluid was Tyrode solution containing glucose (1 mg/ml). The bath was washed by overflow. The suspended segment, called the SCHULTZ-DALE segment (SD segment), was attached to a frontal lever and its contractions were recorded on the kymograph drum. An adjacent strip of ileum or uterus, called the control segment, was immersed in Tyrode solution at 37° C during the experiment. The volumes of added drugs (histamine dihydrochloride or egg albumin, both dissolved in Tyrode solution) were 0.2 ml or less.

In the indicator segment experiments, designed to reveal any release of smooth-muscle stimulating agents into the bath from the SD segment during contraction, a strip of ileum from a non-sensitized guinea pig was suspended in the bath together with the SD segment. Its contractions were recorded by another lever. A similar technique has been used by DWORETZKY (1959).

The experimental procedure was as follows: When both SD strip and indicator strip had relaxed and stable base-lines had been secured, varying doses of histamine were added to the bath in order to obtain dose-response curves. Antigen to a final concentration of 0.004 mg/ml was then added and allowed to remain in the bath for a 3-minute period. Any contraction of the SD strip and indicator strip during this period was estimated in terms of histamine by comparative reference to the dose-response curve for the latter drug. At the end of the 3-minute period the antigen was washed out of the bath. When the SD strip had returned to the initial base-line, it was removed and weighed. A piece of the strip was cut off and placed in the fixative for subsequent mast-cell count. The rest of the strip was weighed, homogenized, and boiled in HCl. After neutralization, the extract was assayed for histamine on atropinized (1.5×10^{-6} M atropine sulphate) guinea-pig ileum. A small piece from the control strip was fixed for histologic examination; the remainder was weighed and homogenized for histamine assay. All histamine values are given as the base.

The histologic treatment comprised fixation, sectioning and staining as described earlier (BORÉUS 1960). For each strip, the total number of recognizable mast cells in four different sections was estimated and expressed as number of cells per square unit of section, the area of which was estimated by means of a square net inserted in the ocular. The mast-cell count of the SD segment was compared with that of the control segment and any depletion of the cell population, i. e., "mast-cell disappearance", was expressed as a percentual decrease from the control segment. (A negative value thus denotes a greater population of mast cells in the SD segment than in the control segment.)

By means of this procedure the following values could be obtained: (1) The degree of SD contraction in terms of histamine; (2) the amount, in terms of histamine, of smooth-muscle contracting substance liberated, over a 3-minute period, from the SD strip into the bath during anaphylactic contraction; (3) the histamine content of the SD strip and of the control strip after the experiment; and (4) the mast-cell disappearance value for the SD strip.

Results

Distribution of mast cells in guinea-pig ileum and uterus. Most of the mast cells in guinea-pig ileum are located in the basal part of the mucosa, in the submucosal layer and, to a somewhat lesser degree, in the circular part of the muscular coat. In counts of 20 different strips, the mast-cell population was 3.2 times higher in the submucosa and the basal third of the mucosa than in the circular muscle layer.

Uterine mucous membrane is poor in mast cells. Only in the deepest layer was some mast-cell accumulation observed. The muscularis mucosae and the submucosa were comparatively rich in mast cells and, in a series of counts in 8 different strips, showed a frequency 2.9 times higher than that of the outer muscular layer.

Several preliminary experiments with "differential" counts had disclosed that the mast cells in different layers of ileum and uterus reacted similarly and in like degree to antigen. In these experiments, therefore, the total numbers of mast cells in whole sections were used for the comparisons.

Experiments with indicator strips in the bath. The principal results are detailed in Table I, and will be reviewed column by column.

(1) The standard antigen concentration of 0.004 mg/ml invariably produced substantial SCHULTZ-DALE contraction in both ileum and uterus, and of like type, intensity and duration. The contraction started after a latency of 10—30 seconds. The maximum was reached after 15—60 seconds, when slow relaxation occurred. The initial base-line was usually reached 10—20 minutes later. It will be seen that the amount of histamine needed to duplicate the reaction was much higher for uterus than for ileum, a finding which is consistent with the well-known difference in the sensitivity of these two tissues to histamine. On occasion the insensitivity of uterus to histamine was so great that the standardization had to be done *after* the anaphylactic reaction in order to avoid too high a concentration of histamine in the bath and possible reduction of the subsequent sensitivity to histamine during the SCHULTZ-DALE reaction, as described by SCHILD (1936). It should be noted that the amount of histamine needed for duplication of the SCHULTZ-DALE contraction in uterus is more than three times the total amount of histamine in the strip.

It was observed that storing of the strips in Tyrode solution at room temperature gradually decreased their ability to contract upon antigen administration but did not produce a commensurable loss of sensitivity to histamine.

Table I. Results of experiments with Schultz-Dale contraction of ileum and uterus in the presence of indicator strip of ileum in the same bath. Final antigen concentration 0.004 mg/ml. Exposure time 3 minutes

Tissue	Number of experiments	1	2		3		4
		Schultz-Dale contraction (in terms of μg histamine)	Liberated contracting substance (in terms of histamine)		Histamine content after experiment		Mast-cell disappearance %
			μg/strip	% of histamine content of the strip	SD strip μg/g	Control strip μg/g	
Ileum ..	9	0.16±0.05	<0.007 ± 0.008	<0.4 ± 0.2	10.2±1.4	10.1±1.4	−7.8 ± 6.6
Uterus	12	1.78±0.39	0.032 ± 0.006	9.3 ± 2.5	7.2±1.4	7.3±1.2	13.0 ± 8.6

(2) Another striking difference between ileum and uterus is evident from the values for the contracting substance released into the bath. This amount, in terms of histamine, was about 5 times higher in the uterus than in ileum. In 5 of the 9 experiments with sensitized ileum, no contraction at all was observed in the indicator strip. In these 5 instances the lower limit for histamine sensitivity was 0.0012 or 0.0018 μg histamine. These limit values were taken as maximal values for liberated contracting substance and are included in the mean values for ileum in column 2 of Table I. This means that the true values for ileum are even lower than 0.007 μg and 0.4 %, respectively.

The contraction of the indicator segment began a few seconds after the onset of the SCHULTZ-DALE contraction and rose slowly to its maximum. When, at the end of the 3-minute period, the bath was washed, the indicator strip immediately relaxed, although the SD strip of ileum or uterus was still in contraction.

(3) Following contraction the total histamine content in the SD strips was not significantly lower than that in the control strips. Assuming, however, that the liberated contracting substance was in fact histamine, a quantity so small would not be demonstrable by comparison of the histamine content of the strips, since the variation of these values is too wide.

(4) It is evident that the variation of the mast-cell disappearance values is wide. Although neither value alone significantly differs from zero, the two values differ from each other ($P = 0.01$—0.05).

A definite difference between the small intestine and the uterus in respect to mast-cell reactivity as well as to histamine liberation on addition of antigen *in vitro* was discovered earlier (BORÉUS and CHAKRAVARTY 1960) when the antigen concentration was 1 mg/ml and the incubation time 10 min. Studies of the mast-cell disappearance during the SCHULTZ-DALE reaction were therefore extended.

Table II. Percentual mast-cell disappearance in ileum and uterus of sensitized and non-sensitized guinea pigs following Schultz-Dale contraction. Exposure time 30 minutes. P values from statistical comparison of sensitized and non-sensitized animals

	Sensitized		Non-sensitized
	Final antigen concentration		
	0.4 mg/ml	4 mg/ml	
Ileum	10.6 ± 4.7 n = 7 P: 0.05 – 0.01	16.0 ± 6.2 n = 4 P: 0.05 – 0.01	– 2.3 ± 2.8 n = 8
Uterus.....	25.5 ± 7.7 n = 6 P: 0.05 – 0.01	34.0 ± 4.7 n = 6 P: < 0.001	– 1.6 ± 5.6 n = 8

In this new series of experiments (without indicator strips), anaphylactic contractions were produced with antigen in final concentrations of 0.4 and 4 mg/ml during a 30-minute period. In order to obviate any possible effect of the muscle contraction itself upon the mast-cell population a series of ileal and uterine strips from non-sensitized animals were suspended in the bath; an adjacent strip serving as a control in the usual way. After maximal relaxation of the strip, antigen (which was shown to have no effect at all on the non-sensitized strip) as well as a suitable dose of histamine were added in order to produce contraction of the strip. The histamine was left in the bath for a sufficient length of time to reproduce the intensity and duration of a SCHULTZ-DALE contraction. (In several experiments additional doses of histamine had to be administered, since histamine contraction is usually of shorter duration than anaphylactic contraction.) The results of all these experiments are summarized in Table II. Mast-cell depletion following the SCHULTZ-DALE reaction was found in both ileum and uterus, although it was comparatively slight. The values were higher with the higher dose of antigen. The uterus showed a stronger mast-cell reaction than the ileum, the difference being most marked at the higher dose. The histamine-produced contractions of the non-sensitized strips gave no mast-cell reaction.

Discussion

SCHILD (1939) showed that different guinea-pig tissues, when incubated with antigen, released different amounts of histamine. Uterus, for instance, released about 20 times as much histamine as ileum, which was very resistant to antigen. A similar discrepancy between uterus and jejunum was found by BORÉUS and CHAKRAVARTY (1960). They pointed out that incubation of jejunum was

followed by the appearance of very small amounts of histamine, no "slow reacting substance" (SRS, a lipid-soluble smooth-muscle stimulating principle) and no mast-cell disappearance although the content of both histamine and mast cells was high. Incubation of uterus, on the other hand, resulted in anaphylactic appearance of histamine and SRS and in disappearance of mast cells.

A similar difference is now demonstrated during the SCHULTZ-DALE reaction despite the apparent similarity in onset, intensity and duration of the contraction. Thus, much higher amounts of smooth-muscle contracting substance was liberated from uterus than from ileum. Moreover, with higher antigen doses the observed mast-cell disappearance was more conspicuous in uterus than in ileum.

Is liberation of contracting agents from tissue cells essential to the anaphylactic contraction? Assuming that such liberation is the direct cause (and not the result) of the contraction and that a part at least of the liberated substance is histamine, it may be asserted on the basis of the results, that the small amount released in ileum is compensated by the high sensitivity of the muscle cell to this drug, and that the greater release in uterus corresponds to a lesser sensitivity.

This investigation was not designed to study the nature of the released agents. The results cannot settle the question whether histamine is of major importance in the SCHULTZ-DALE reaction, since neither pharmacologically induced duplication of the anaphylactic contraction nor quantitative determination of released smooth-muscle stimulating principles can help to establish the precise concentration of these agents at receptor sites within the tissue. Several authors, however, have called attention to numerous discrepancies in the histamine theory of the SCHULTZ-DALE reaction (SCHILD 1936, 1939, FELDBERG 1941, MAYER 1950, HAWKINS and ROSA 1956, REUSE 1956, DWORETZKY 1959). It must be pointed out that the arguments for or against the role of histamine in the reaction are based upon the assumption that the receptors involved are the same whether histamine is liberated in the tissue or whether it is added to the bath solution.

Other agents which have been ascribed a role in the SCHULTZ-DALE reaction are acetylcholine and serotonin (NAKAMURA 1941, DANIELOPOLU *et al.* 1948, GEIGER, HILL and THOMPSON 1956). On the basis of experiments with various inhibitors, GEIGER and ALPERS (1959) concluded that at least three humoral agents, histamine, acetylcholine and serotonin, are involved in the reaction. Other investigators, however, have not suceeded in demonstrating the release of serotonin in the anaphylactic contraction of guinea-pig smooth muscle (FINK and GARDNER 1958; BORÉUS and WESTERHOLM 1961). Though SRS could not be detected after incubation of the small intestine with antigen, on incubation of uterus it occurred in amounts closely corresponding to those of histamine (BORÉUS and CHAKRAVARTY 1960). Quantitative evaluation of the role of SRS and of other agents is an intricate problem since it must be assumed that

they may interact. For instance, BROCKLEHURST (1956) as well as CHAKRAVARTY, HÖGBERG and UVNÄS (1959) showed that SRS sensitized isolated guinea-pig ileum to histamine.

It is possible that the anaphylactic contraction is the result of a combination of antigen and antibody in or on the muscle cell itself. Further elucidation of the SCHULTZ-DALE reaction will require more study of the response to antigen of the smooth-muscle cell itself. LEHOTAN and HASÍK (1959) have shown that, on addition of antigen, actomyosin fibers from smooth muscle of sensitized guinea pigs exhibit contraction analogous to that associated with the SCHULTZ-DALE reaction. Their experiments on this "nerve-free model of muscle" indicate that liberation of contracting substances from other cells (such as mast cells) may not be essential to the anaphylactic contraction of the smooth-muscle cell.

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Studies on the Peripheral Circulation and Metabolism in Man

I. Oxygen utilization and lactate-pyruvate formation in the legs at rest and during exercise in healthy subjects

By

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Abstract

CARLSON, L. A. and B. PERNOW. *Studies on the peripheral circulation and metabolism in man. I. Oxygen utilization and lactate-pyruvate formation in the legs at rest and during exercise in healthy subjects.* Acta physiol. scand. 1961. *52.* 328—342. — Arterial and femoral venous oxygen and lactate-pyruvate was studied at rest, during and after leg exercise in 15 healthy human males. At rest in the supine position the femoral venous oxygen saturation was 53—81 per cent. A few minutes after starting work, the venous oxygen saturation was 22—42 per cent and a slight but steady decrease was observed on continued work. Five minutes after work, the venous oxygen saturation was significantly higher than at rest before work. The pH of the femoral venous blood decreased from mean 7.41 to 7.25 at maximal load. The venous lactate rose during exercise to 4.45—8.66 mM/liter and the venous-arterial lactate difference to mean 0.85 mM/liter at a heart rate of 160—187/minute. After exercise the lactate decreased successively in most cases but had even 30 min later not reached the initial level before work. The venous pyruvate concentration was 0.09—0.15 mM/liter at rest, and increased successively during exercise to 0.22—0.35 mM/liter at the highest loads. A further increase in pyruvate was obtained during the first minutes after work. The increase in "excess of lactate", as calculated from the lactate-pyruvate ratio, during exercise was practically parallel to that in total lactate, indicating that during work the total lactate is a good indicator of the adequacy of oxygen supply to the

tissues. After exercise, however, there was a discrepancy between the total and excess of lactate, the latter decreasing much more rapidly. No difference was obtained between older and younger subjects concerning increase in pulse rate or changes in oxygen saturation and blood lactate-pyruvate during exercise. After work lactate decreased more slowly in the older subjects than in the younger.

Studies on the metabolism of resting and exercising tissues are greatly facilitated by the supply of arterial as well as venous blood draining the tissue, which permits analyses of arteriovenous (AV) differences of the different metabolites. In man this technique has been successfully used in studies of the metabolism of heart muscle *in vivo* by BING and co-workers (for references, see BING *et al.* 1954). However, for many reasons it has not been applied to the same extent in studies on the metabolism of peripheral muscles. DONALD *et al.* (1957) studied the femoral venous oxygen saturation and AV oxygen difference at rest and during supine leg exercise. The AV oxygen difference was found to increase almost linearly with increasing work, and to be closely related to the cardiac output. The calculated blood flow through the exercising leg increased about ten-fold with heavy work. Simultaneously, the blood flow through resting tissue was markedly reduced (BISHOP *et al.* 1957). In a previous study (CARLSON and PERNOW 1958), we were able to confirm the results of DONALD *et al.* (1957). The venous oxygen saturation was, at the beginning of light exercise, reduced to between one-half and one-third of that at rest in the supine position, whereas continuous work caused only a slight further decrease. During exercise performed with one leg only, the venous oxygen saturation decreased at a similar rate in the resting leg as in the exercising one.

The increase in blood lactate concentration has been widely used as an index of the degree of anaerobic metabolism in exercising tissue (for references see HOLMGREN and STRÖM, 1959). During exhaustive work, a ten-fold increase in blood lactate has been repeatedly observed (ASMUSSEN 1950, ÅSTRAND 1952) and in acute or chronic hypoxia either experimentally induced or in congenital heart disease the blood lactate has been found to increase at a lower working intensity than otherwise (DILL *et al.* 1931, LUNDIN and STRÖM 1947). Determination of the AV lactate difference gives the most reliable information about the adequacy of the oxygen supply to the working muscles. This was evident from a preliminary study, in which the AV oxygen and lactate differences in exercising legs of patients with impaired peripheral circulation were compared to corresponding values in healthy subjects (CARLSON and PERNOW 1959).

An accumulation of blood lactate is, however, observed not only in muscular work, but also in conditions where hypoxia is not present, for example after hyperventilation and administration of glucose, epinephrine or pyruvate (for references see HUCKABEE 1958 a). Consequently lactate alone is not a definite index of the adequacy of cellular oxygenation. In all the aforementioned conditions (except hypoxia) in which lactate accumulates, a simultaneous

increase takes place in pyruvate (HUCKABEE 1958 a). With an inadequate oxygen supply, reoxidation of the reduced diphosphopyridine nucleotide (DPNH) to its oxizidized form (DPN) — necessary for the carbohydrate breakdown to form high energy phosphate bonds — is channelled by reduction of pyruvate to lactate. Hypoxia therefore results in an increased formation of lactate from pyruvate (ASMUSSEN 1950). As a measure of the degree of oxygen deficiency, HUCKABEE (1958 a) used the calculation of "excess of lactate" (XL), *i. e.*, the lactate change exceeding that which is directly proportional to pyruvate changes.

The results obtained with the peripheral catheterization technique will be described in a series of papers. Our main purpose has been to study the degree of oxygen utilization and the lactate pyruvate formation in the legs during exercise in the healthy state, as well as in conditions characterized by a limitation of the working capacity. It was hoped that this technique would provide a measure of the degree of aerobic and anaerobic work in the muscular tissues. The present paper comprises data from healthy subjects, 10 young and 5 older men, and forms the basis for comparison in the pathologic states presented in subsequent papers (CARLSON and PERNOW, to be published).

Case Material

The case material consisted of two groups of healthy male volunteers, 10 aged 20—37 years, and 5 aged 48—56 years. All were clinically examined for cardiovascular and pulmonary function and found to be healthy.

Methods

The investigation was carried out on fasting subjects in the morning. The brachial artery and femoral vein were punctured percutaneously, and polyethylene catheters inserted by a technique described earlier (BERNEUS *et al.* 1954). The femoral vein was punctured about 2 cm below the inguinal ligament, and the catheter inserted about 8 cm distally. Some minor hematomas were the only complication.

Exercise was performed in a sitting position on a bicycle ergometer, and consisted of an uninterrupted series of work loads, starting with 150—300 kpm/minute and increasing every 5 min by 150—300 kpm/minute. The work was performed with one leg only, the pedal being fitted with a spring, to make its return passive. The one-leg exercise was chosen, since the subjects were also used as controls in another series of experiments, in which the same study was made in patients with unilateral circulatory disturbances of leg (CARLSON and PERNOW, to be published). The heart rate was calculated from the ECG.

The room temperature during the investigations was 20—23° C, and no arrangement was made to keep it constant.

Arterial and venous blood samples were drawn simultaneously at rest, as well as once at each work load during exercise, and several times within 30 min of ending work. The blood was drawn into 20-ml glass syringes, their dead space being filled with one per cent heparin solution. All resting values refer to the supine position, and those obtained during exercise to the sitting position.

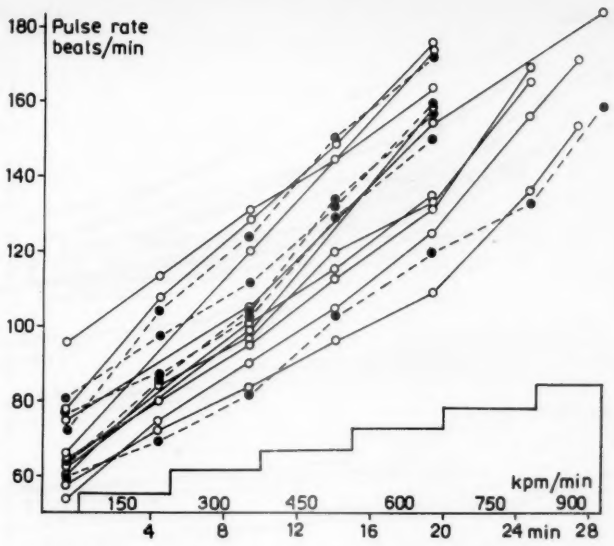

Fig. 1. Pulse rate at rest and at different loads during one-leg exercise with successively increasing load. ○——○ = young men; ●– – –● = older men.

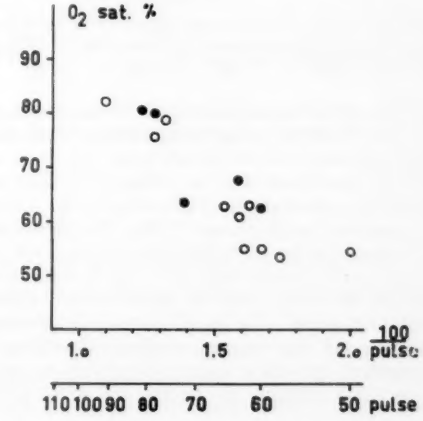

Fig. 2. Femoral venous oxygen saturation (%) at rest correlated to pulse rate (beats/min) at rest. o = young men; ● = older men. Venous O_2 saturation $= -37.1 \cdot \frac{100}{\text{pulse}} + 124.4$ ($p < 0.001$).

Duplicate estimates of the oxygen saturation and hemoglobin concentration were made spectrophotometrically, the former by a slight modification of the method described by DRABKIN (1950). The oxygen content was calculated from the oxygen saturation and the oxygen capacity of the blood sample. All these determinations were made on each blood sample. The error of the methods as used in this laboratory has recently been reported (HOLMGREN and PERNOW 1959).

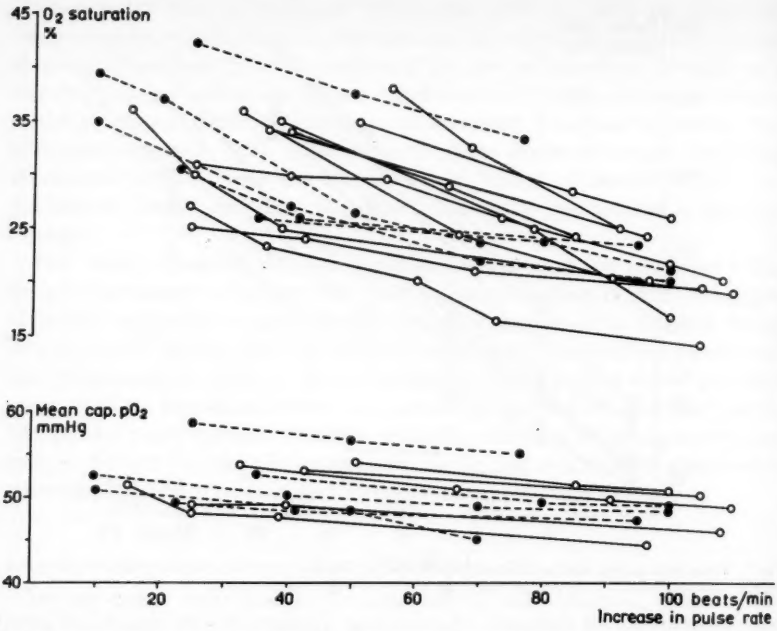

Fig. 3. Femoral venous oxygen saturation in % (upper diagram) and calculated mean capillary pO_2 in mm Hg (lower diagram) during one-leg exercise, correlated to rise in pulse rate from the resting value. Symbols as in Fig. 1.

The pH of whole blood at 37° C was measured by a glass electrode and a potentiometric pH-meter, using phosphate buffer as standard. The standard error of a single determination was 0.002 pH units. Blood lactic acid was determined with STRÖM's (1949) modification of the colorimetric method of BARKER and SUMMERSON (1941). Pyruvate was determined according to the method of FRIEDMANN and HAUGEN (1943), as modified by HUCKABEE (1956). The determinations were always made in duplicate. The precipitation of the blood by trichloracetic acid for lactate and pyruvate determinations was performed within 15 sec of withdrawing the blood. In preliminary experiments, the resting value for blood pyruvate obtained with this method was compared with that given by the paper chromatography method of SELIGSON and SHAPIRO (1952). Exactly the same values were obtained with the two methods. The error of the method (standard error of a single determination) calculated from duplicate determinations, was 0.013 mM/liter for lactate, and 0.005 mM/liter for pyruvate.

Results

Heart rate in relation to work loads

In every case, a linear relation existed between the increase in heart rate and the work load (Fig. 1). The pulse rate at rest, at the different work loads and after work, did not differ significantly in the older subjects from that in the younger ones.

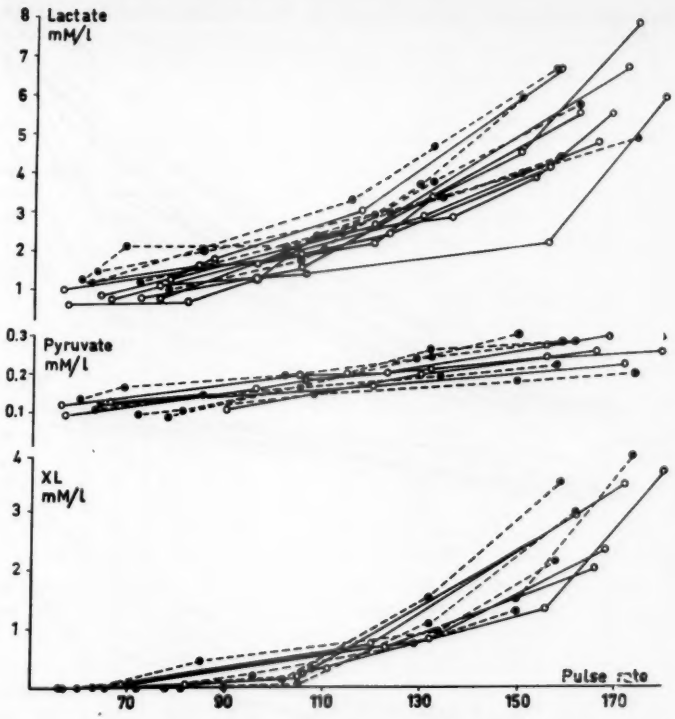

Fig. 4. Arterial lactate, pyruvate and "excess of lactate" (XL) in mM/liter, at rest and during exercise, correlated to relative work intensity (pulse rate during work). Symbols as in Fig. 1.

Oxygen saturation of arterial and venous blood

The oxygen saturation of arterial blood was in all cases 96—99 per cent at rest, and unchanged during and after work. At rest in the supine position the oxygen saturation of the femoral venous blood was 53—81 per cent (mean 69), giving an AV oxygen difference of 23—85 ml/liter (mean 49). The venous oxygen saturation at rest was in good correlation to the pulse rate (Fig. 2). During the first load of exercise, a venous oxygen saturation of 22—42 per cent (mean 30) was obtained at a heart rate of 68—102/min (mean 81). On continued exercise with successively increasing loads, a further slight but steady decrease in venous oxygen saturation occurred in every case, the lowest values always being recorded at the highest load, with minimal values of 13—34 per cent saturation (mean 21), at a heart rate of 159—187/min (mean 169) (Table I and Fig. 3).

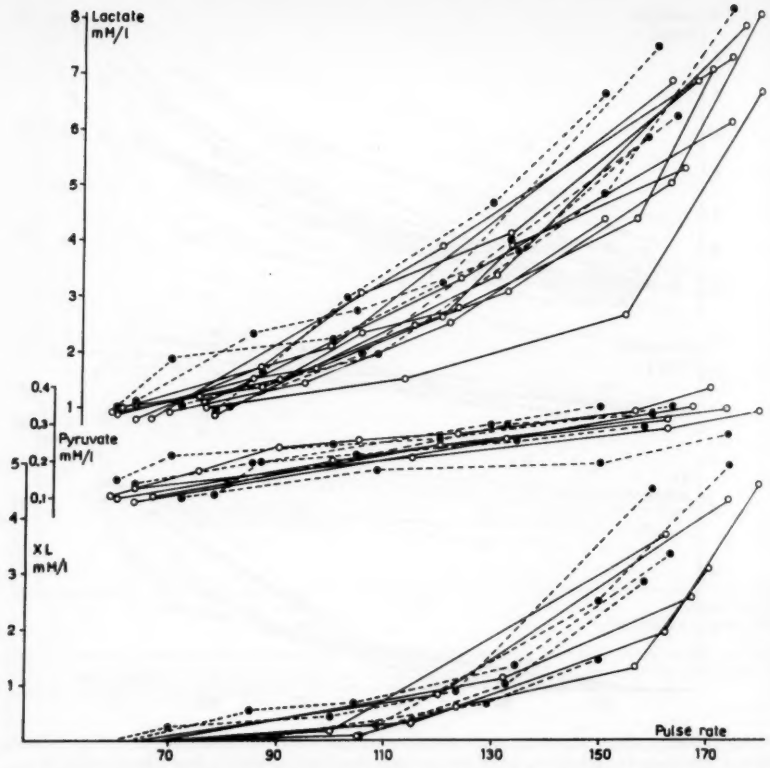

Fig. 5. Femoral venous lactate, pyruvate and "excess of lactate" (XL) in mM/liter, at rest and and during exercise. Symbols as in Fig. 1.

The venous oxygen saturation increased rapidly after work to values exceeding those at rest before work. Thus, the venous oxygen difference determined in the supine position 5 min after the end of exercise was 70—89 per cent (mean 79), the value being significantly higher than before work ($p < 0.001$). The initial level before exercise was regained about 15 min after the end of exercise.

There was no difference between the two age groups with respect to arterial or venous oxygen saturation at rest, during or after work.

pH

The pH of the femoral venous blood decreased during exercise from 7.41 at rest to 7.24 at maximal load (mean values). The corresponding values for arterial blood were 7.43 and 7.37. Still 5 minutes after work, the pH of both arterial and venous blood was lower than before work.

Table I. Data obtained in connection with leg exercise in normal human subjects

		Rest	Heart rate during exercise				After exercise			
			90–110	110–130	130–150	150–175	5 min	10 min	15 min	30 min
Venous oxygen saturation per cent	N	15	14	9	13	13	9	9	9	
	M	69	30	29	23	22	79	71	68	
	SE	2.1	1.4	1.9	0.8	1.0	1.8	2.8	2.8	
	SD	8.0	5.6	5.8	1.8	4.0	5.5	7.9	8.3	
Arterial pH	N	10	7	4		10	6			
	M	7.432	7.409	7.386		7.369	7.359			
	SE	0.004	0.008			0.011	0.011			
	SD	0.010	0.017			0.026	0.022			
Venous pH	N	10	7	4	5	10	7			
	M	7.416	7.343	7.327	7.297	7.239	7.311			
	SE	0.008	0.015			0.011	0.020			
	SD	0.021	0.038			0.026	0.052			
Venous pO_2	N	10	7	4		10	7			
	M	37	25	23		20	45			
	SE	1.1	0.7			0.6	1.7			
	SD	3.3	1.8			1.5	5.1			
Arterial lactate mM per liter	N	15	9	7	10	13	6		4	
	M	0.97	1.71	2.60	3.62	5.61	4.84		2.70	
	SE	0.05	0.10	0.12	0.18	0.41	0.42			
	SD	0.15	0.26	0.37	0.52	1.21	1.05			
Venous lactate mM per liter	N	15	11	9	10	15	10	8	10	9
	M	0.97	2.10	2.91	3.78	6.56	6.29	5.42	3.93	2.61
	SE	0.03	0.17	0.25	0.18	0.42	0.55	0.40	0.33	0.25
	SD	0.11	0.58	0.83	0.46	1.24	1.75	1.14	1.06	0.76
Arterial pyruvate mM per liter	N	10	10		6	10	10			
	M	0.11	0.18		0.21	0.25	0.39			
	SE	0.01	0.01		0.02	0.01	0.03			
	SD	0.02	0.02		0.04	0.03	0.09			
Venous pyruvate mM per liter	N	10	10		6	10	10	9	10	8
	M	0.12	0.22		0.27	0.31	0.45	0.43	0.35	0.21
	SE	0.01	0.01		0.01	0.02	0.03	0.02	0.02	0.01
	SD	0.02	0.03		0.05	0.06	0.10	0.07	0.05	0.04
Arterial XL mM per liter	N		7	7		10	10			
	M	0	0.16	0.58		2.84	2.05			
	SE		0.04	0.06		0.27	0.43			
	SD		0.09	0.15		0.86	1.32			
Venous XL mM per liter	N		9	8		10	10	9	9	8
	M	0	0.26	1.03		3.16	2.18	1.11	0.64	0.27
	SE		0.05	0.07		0.31	0.46	0.28	0.23	0.08
	SD		0.15	0.20		1.10	1.47	0.84	0.70	0.20

Abbreviations: N, number of observations; M, mean; SE, standard error of the mean; SD, standard deviation.

Blood oxygen tension

The oxygen tension of arterial and venous blood was calculated in 10 cases from the HbO_2 and pH, using the diagram of DILL, EDWARDS and CONSOLAZIO (1937). The resting value for arterial blood calculated on this basis was 85—115 mm Hg (mean 96). During light and moderate work, no significant change in arterial pO_2 was observed. At the end of work, however, a slight decrease in the mean value to 90 mm Hg was observed, which agrees with earlier observations made with direct methods (HOLMGREN and LINDERHOLM 1958). The resting value for femoral venous pO_2 was 33—47 mm Hg (mean 37). A steady decrease in venous pO_2 to 20—25 mm Hg was found during the first two loads; thereafter, an essentially steady level was maintained during continued exercise (Table I). Five minutes after the end of work, the venous pO_2 had increased to 33—55 mm Hg (mean 47). The values for the mean capillary pO_2 during exercise, as calculated from BARCROFT's (1934) formula:

$$\text{ven. } pO_2 + \frac{\text{art. } pO_2\text{-ven. } pO_2}{3}$$ are plotted in Fig. 3.

Arterial and venous lactate concentration

No significant difference could be demonstrated at rest between the arterial and femoral venous lactic acid concentration, when calculated on the individual differences (0.75—1.23 mM/liter, mean 0.97, and 0.79—1.25 mM/liter, mean 0.97, respectively). As seen in Fig. 4 and 5 and Table I, a steady rise in both arterial and venous lactate was observed during the first loads, with a more pronounced increase at the highest load, at which lactate concentrations of 4.35—7.73 mM/liter (mean 5.71) and 4.45—8.66 mM/liter (mean 6.56) respectively, for arterial and femoral venous blood were recorded. This arteriovenous lactate difference is significant ($p < 0.01$).

Five minutes after work the venous lactate concentration had further increased compaired to the levels at the end of work in 3 cases, was constant in 3 cases and had slightly decreased in further 4 cases (Fig. 6). The arterial lactate generally decreased more rapidly than the venous lactate. Five minutes after work, the mean arterial and venous lactate concentration was 4.84 and 6.29 mM/liter, respectively, and after 15 min 2.60 and 3.75 mM/liter, respectively. These differences are highly significant ($p < 0.001$). There was no relation between the lactate concentration at maximal exercise and that 5 min after work; a successive decrease in lactate was observed in cases with both high and low concentration at the end of work (Fig. 6).

Arterial and venous pyruvate concentration

Pyruvic acid concentration at rest was 0.08—0.15 mM/liter (mean 0.11) in arterial blood, and 0.09—0.15 mM/liter (mean 0.12) in femoral venous blood and no significant AV difference was found. During exercise, a successive increase was observed in both arterial and venous blood, with a maximal value

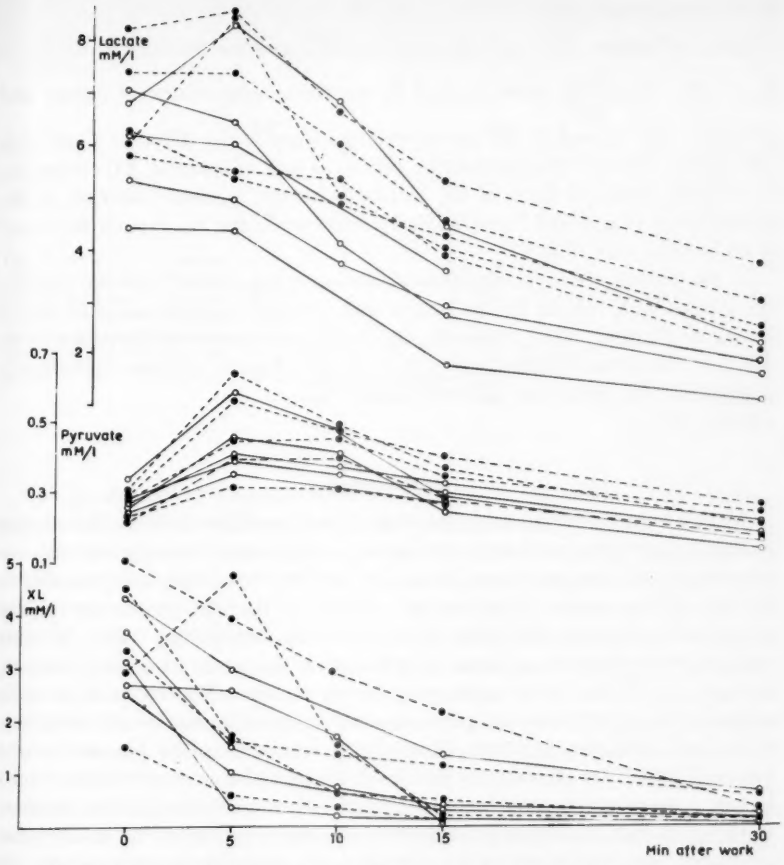

Fig. 6. Femoral venous lactate, pyruvate and XL at maximal work performed (time 0) and after exercise. Symbols as in Fig. 1.

of 0.33 mM/liter in venous blood at 900 kpm/minute. At the highest loads, the mean arterial and venous pyruvate concentrations were 0.25 and 0.31 mM/liter respectively, the difference being significant ($p < 0.01$). When work stopped, the arterial and venous pyruvate increased further, with a maximum reached 5 min after cessation of work at which moment a mean venous pyruvate of 0.45 mM/liter was obtained. It then decreased successively, and 30 min after work had fallen to about the same level as at the end of work (Fig. 6). During the first 30 min after the end of work the AV difference in pyruvate concentration was significant ($p < 0.01$).

Blood lactate-pyruvate ratio.

Excess of lactate (*XL*) was calculated according to the formula $X_L = (L_n - L_o) - (P_n - P_o)\left(\frac{L_o}{P_o}\right)$, where L_o and P_o are the resting values for lactate and pyruvate, and L_n and P_n the corresponding values at the different work loads (HUCKABEE 1958 a). During exercise, venous as well as systemic XL concentration closely followed those of the lactate, with the maximal increase at the highest loads (Fig. 4 and 5 and Table I). After work, the XL rapidly decreased in all but one case (Fig. 6).

In the present series, no significant difference was present between the two age groups with respect to the lactate and pyruvate concentration at rest or during work. After work, however, the lactate concentration decreased more slowly in the older subjects than in the younger (Fig. 6), whereas interestingly enough no such trend was observed for the XL.

Discussion

With the technique used in this study, it was possible to follow the oxygen utilization and the production of lactate and pyruvate during exercise. As pointed out in a previous paper (CARLSON and PERNOW 1959), it is probable — in view of the position of the venous catheter — that the greater part of the blood collected originated from muscle, at any rate during work. All data obtained during work have been plotted against the relative working intensity, as expressed as the rise in pulse rate during exercise. This seems to be most adequate, since differences in physical working capacity can hereby be largely eliminated. This fact has most clearly been demonstrated by HOLMGREN and STRÖM (1959). They showed that patients with heart failure had a higher blood lactate concentration than healthy subjects when correlated to the absolute work (work load), whereas no difference was obtained when the lactate concentration was correlated to the relative work intensity (heart rate). In the present series, no significant difference was observed between subjects of various age with respect to the rise in heart rate during work (Fig. 1).

The femoral venous oxygen saturation at rest ranged from 53 to 81 per cent, and was in linear relation to the heart rate (Fig. 2) with the highest saturation at the highest heart rate. Since the oxygen uptake in the legs is probably constant at rest, the AV oxygen difference is inversely correlated to the pulse rate. Consequently, at a high heart rate, which normally indicates a large cardiac output, either a smaller relative amount of oxygen is taken up in the muscles, or a greater part of the blood flows through non-muscular tissues. The oxygen saturation of venous blood probably reflects the distribution of the blood flow through different tissues, rather than being a measure of the metabolic state (PAPPENHEIMER 1941).

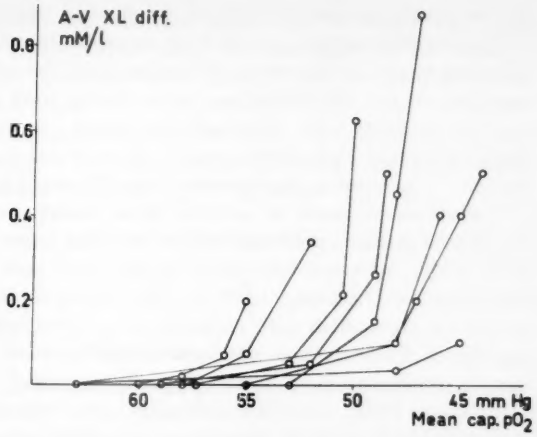

Fig. 7. Difference between "excess of lactate" (XL) in femoral venous and arterial blood, at rest and during exercise, correlated to mean capillary pO_2. Symbols as in Fig. 1.

Already during the first minutes of the initial load, the venous oxygen saturation had decreased to 22—42 per cent. This decrease is, however, obviously not due entirely to the exercise, since a considerable decrease in oxygen saturation is produced merely by changing the position from supine to sitting (FLORKIN, EDWARDS and DILL 1940). This was confirmed in three cases in this series, in which the femoral venous saturation decreased from 65—73 to 24—31 per cent merely by changing the position from supine to sitting. The further decrease during exercise was small. These changes at rest were obviously due to a variation in blood flow, which has been shown to be markedly reduced when changing the body posture from supine to sitting (BEACONSFIELD and GINSBURG 1955). In two healthy subjects (not included in this series), two leg exercises were performed in the recumbent position. An immediate fall in femoral venous oxygen saturation was observed during work. The changes in the oxygen and lactate concentration in the blood were similar to those in sitting exercise, when correlated to the increase in heart rate. As shown in a previous paper (CARLSON and PERNOW 1959), a simultaneous decrease in venous oxygen saturation was also observed in resting tissues during one-leg exercise. It was supposed that this decrease, which was of about the same magnitude as in the exercising tissue, reflected a decrease in blood flow through resting tissues during exercise.

It is interesting to note that the oxygen utilization was almost similar at different working intensities. Even during heavy work (heart rate up to 187/minute) the blood leaving the exercising leg was saturated to 21 per cent (mean value). It is not known whether more complete utilization takes place during maximal work. The fact that the femoral venous blood contains considerable oxygen even on heavy work might be due either to the possibility

that a certain amount of blood is still flowing through non-muscular tissues, or to incomplete oxygen uptake from muscular blood. As will be shown in the following paper in this series (CARLSON and PERNOW, in press), practically complete oxygen utilization may occur during work under certain conditions, *i. a.*, in patients with impaired peripheral circulation. Fig. 7 shows that, despite the fairly high oxygen tension of the blood on heavy work, the aerobic capacity is incomplete, as is evident from the rapid increase in XL formation.

The values obtained for arterial blood lactate were of the same order of magnitude as those presented earlier with the same analytic method (GOLDSMITH 1948), whereas even lower values have been reported by others (for references see HUCKABEE 1958 a). The values obtained for arterial pyruvate are also in agreement with earlier reports (GOLDSMITH 1948 and others). No significant difference was obtained at rest between the arterial and venous lactate concentration. This also applied to arterial and mixed venous blood (HOLMGREN 1959), whereas significantly higher values were observed in venous muscular blood of the forearm than in arterial samples (ANDRES, CADER and ZIERLER 1956). No significant difference was found between young and old subjects with regard to arterial or venous lactate concentration at rest or during exercise, which conforms with earlier observations (HOLMGREN and STRÖM 1959). In larger series, however, a higher arterial lactate concentration in relation to heart rate during work has been observed in older healthy subjects than in younger ones (ÅSTRAND 1960). Obviously, the similarity in rate of lactate accumulation, as well as of oxygen consumption and AV oxygen difference at different ages found in this study might indicate a similar increase in regional blood flow during exercise. A comparable blood flow through the calf at rest and immediately after work has, in fact, been found in young and older healthy subjects with the plethysmographic technique (ALLWOOD 1958).

After work, the lactate concentration decreased more slowly in the older subjects than in the younger (Fig. 5). This fact cannot, however, be taken as proof of prolonged tissue hypoxia, since the same difference between the two age groups was observed with respect to pyruvate. The fall in XL factor after work was the same in the older subjects as in the younger ones (Fig. 6).

As mentioned in the introduction, the lactate alone is not a definite index of the adequacy of cellular oxygenation, since an increase in lactate is also observed on other occasions where the oxygen supply of the tissues is not decreased. The onset and degree of anaerobic work are best studied by simultaneous estimation of lactic and pyruvic acid concentrations in the blood (HUCKABEE 1958 a, b). During work, lactate alone is markedly raised in the arterial blood, whereas pyruvate is only slightly (ASMUSSEN 1950, HUCKABEE 1958 b), which — as shown in this study — also applies to venous blood from exercising tissue. Consequently, the curves for total lactate and excess of lactate run practically parallel (Fig. 4 and 5). During work, blood lactate determinations can therefore normally be regarded as a specific test of the effectivity of oxygen supply

to the tissue. This is not, however, the case after work. As seen from Fig. 6, the venous blood lactate either further rose, was unchanged or fell slowly 5 min after the end of exercise. This observation must not be taken as an indication of a prolonged state of tissue hypoxia. It was found that the pyruvate concentration consistently increased immediately after work, and that the lactate-pyruvate ratio therefore rapidly decreased, which is in conformity with earlier observations on arterial blood (ASMUSSEN 1950, HUCKABEE 1958 b).

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Further Studies on the Effect of Catechol on the Isolated Guinea-Pig Ileum

By

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Abstract

SJÖSTRAND, N. O. *Further studies on the effect of catechol on the isolated guinea-pig ileum.* Acta physiol. scand. 1961. *52.* 343—349. — Catechol in doses of 0.1—2 mg added to the isolated guinea-pig ileum in a 15 ml bath usually induced a contraction of the strip. Atropine and paralyzing doses of cocaine and hexamethonium abolished this contraction. BOL and phenbenzamine had no effect on contractions due to catechol. Catechol in non-stimulating doses reduced the inhibition caused by adrenaline and noradrenaline. It is suggested that catechol like nicotine stimulates the gut through the mediation of acetylcholine and stimulation of the intramural ganglia.

Catechol has been found as a natural constituent of human urine (FIKER 1953, EULER and LISHAJKO 1959) and may therefore occur in some tissues and exert local physiological actions. In this respect the findings of BACQ (1936) that catechol exerts a sensitizing action on the effect of adrenaline, probably due to inhibition of the catechol o-methyltransferase (BACQ *et al.* 1959) are of great interest.

In a previous report the effect of catechol on the isolated guinea-pig ileum has been studied (SJÖSTRAND 1960). It was found to induce contraction of the ileum, when doses of 0.5—5 mg/15 ml were used, and to cause relaxation of the strip when bigger doses were used. Stimulation of plain muscles due to catechol has earlier been described by BARGER and DALE (1910—11), who suggested a direct effect on the smooth muscle fibres. Since in the previous report the catechol induced contraction was abolished by atropine and by paralysing doses of

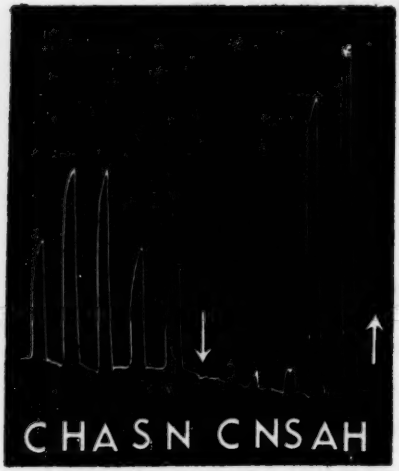

Fig. 1. Isolated guinea-pig ileum; bath volume 15 ml.
A = 0.01 μg acetylcholine, C = 0.5 mg catechol, H = 0.1 μg histamine, N = 5 μg nicotine, S = 0.3 μg serotonin. Between arrows 150 μg cocaine in the bath.

nicotine, the conclusion was drawn that catechol exerts its stimulating property on the isolated guinea-pig ileum through the mediation of acetylcholine and stimulation of the intestinal ganglia. The present study was undertaken in order to obtain further information on the above mentioned effect and to examine whether catechol could modify the actions of adrenaline and noradrenaline on the guinea-pig ileum.

Materials and methods

Young guinea-pigs weighing 150—250 g were killed by a blow on the head. A strip of about 2 cm was taken from the caudal part of the ileum and fixed in a 15 ml organ bath containing Tyrode solution aerated with oxygen and kept at 38° C. The contractions were recorded with a linear frontal writing lever.

The solutions used were acetylcholine chloride 0.01—10 μg per ml, adrenaline hydrochloride 1C—100 μg per ml, 2-brom-lysergic acid diethylamide (BOL) 50—500 μg per ml, catechol 1—10 mg per ml, cocaine hydrochloride 10 mg per ml, hexamethonium hydrobromide 1—10 mg per ml, histamine dihydrochloride 0.1—10 μg per ml, nicotine bitartrate 0.1—1 mg per ml, noradrenaline hydrochloride 10—100 μg per ml, phenbenzamine (Lergitin) 0.1—1 mg, serotonin creatinine sulphate 1—10 μg per ml and substance P 10 units per ml.

When the effect of catechol on the action of adrenaline and noradrenaline was examined the following procedure was used. A dose of acetylcholine, histamine or substance P, giving steady responses was chosen, then the maximal dose of catechol that had no effect by itself or on the test dose was found. When this was done a dose of adrenaline or noradrenaline giving slightly more than fifty per cent decrease of the response to the test dose was given this dose was then given together with the catechol dose and the effect on the test dose was examined. The test doses were given with an

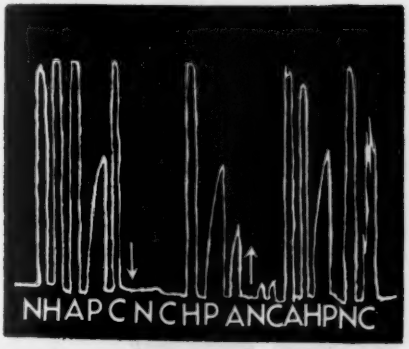

Fig. 2. Isolated guinea-pig ileum; bath volume 15 ml.
A = 0.03 μg acetylcholine, C = 0.25 mg catechol, H = 0.2 μg histamine, N = 10 μg nicotine, P = 0.5 units substance P. Between arrows 300 μg hexamethonium in the bath. About 5 minutes between each contraction.

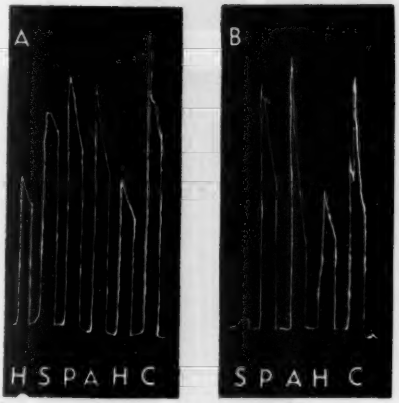

Fig. 3. Isolated guinea-pig ileum; bath volume 15 ml.
A = 0.01 μg acetylcholine, C = 0.5 mg catechol, H = 0.01 μg histamine, P = 0.5 units substance P, S = 0.5 μg serotonin.
A. before BOL
B. 10 μg BOL in the bath.

interval of 5 min and the doses of adrenaline, noradrenaline and catechol were added to the bath one minute before the test doses.

In each series of experiment 5—10 experiments with intestines from 3—6 different animals were performed.

Results

Catechol in doses of 0.1—2 mg added to the 15 ml organ bath usually induced a contraction of the gut. The contraction often showed tachyphylaxis, which generally could be avoided if the intestine was allowed to rest for about 5 min between each catechol induced contraction. In some cases the earlier described "after effect" (SJÖSTRAND 1960) was seen.

Cocaine (10—50 μg/ml) inhibited the contraction due to catechol as well as the contractions due to nicotine (5—20 μg) and serotonin (0.05—5 μg) while

Fig. 4. Isolated guinea-pig ileum; bath volume 15 ml. A = 0.01 μg acetylcholine, C = 0.1 mg catechol, H = 0.01 μg histamine.
A. before phenbenzamine (Lergitin)
B. 10 μg phenbenzamine in the bath.

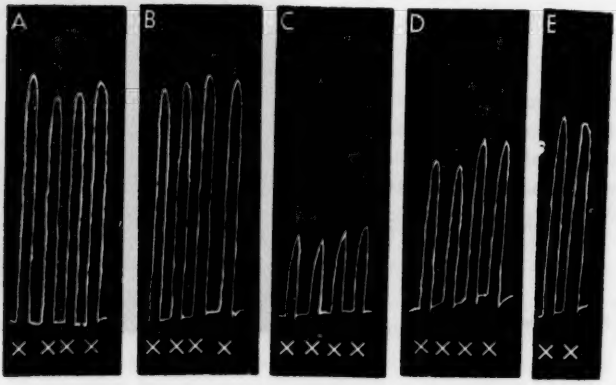

Fig. 5. Isolated guinea-pig ileum; bath volume 15 ml.
At × addition of 0.01 μg acetylcholine. 5 minutes between each contraction.
A. before adrenaline and catechol.
B. 20 μg catechol in the bath 1 minute before contraction.
C. 1 μg adrenaline 1 minute before contraction.
D. 1 μg adrenaline and 20 μg catechol 1 minute before contraction.
E. after adrenaline and catechol.

the effects of histamine, acetylcholine and substance P remained unaltered or were augmented (Fig. 1).

Hexamethonium (5—200 μg/ml) obliterated the effect of catechol and that of nicotine. The effects of substance P, serotonin and histamine were unaffected or increased after addition of hexamethonium. The effect of acetylcholine was increased, unchanged or reduced (Fig. 2).

BOL in doses (5—50 μg) sufficient to greatly reduce the action of sero-

tonin (0.1—5 μg) had no effect on contractions caused by catechol, acetylcholine, histamine and substance P (Fig. 3).

Phenbenzamine (Lergitin) in doses of 1 to 10 μg, which depressed the effect of histamine (0.01—1 μg) while leaving those of acetylcholine and substance P unimpaired, had no effect on contractions due to catechol (Fig. 4).

Catechol in a non-stimulating dose (10—50 μg) added together with a depressing dose of adrenaline or noradrenalin (1—5 μg) reduced the inhibiting action of adrenaline and noradrenaline upon contractions caused by acetylcholine, histamine and substance P (Fig. 5). When catechol was added to the bath together with adrenaline or noradrenaline there was sometimes seen an increase in the basal tone of the strip.

Discussion

It has been shown in the present study that the stimulating action of catechol on the isolated guinea-pig ileum can be abolished by cocaine and hexamethonium but not by BOL or phenbenzamine (Lergitin).

From the previous experiments (SJÖSTRAND 1960) it was suggested that catechol does not exert a direct tonic effect on the smooth muscle fibres as assumed by BARGER and DALE (1910—11), but stimulates the gut through liberation of acetylcholine mediated by the intramural ganglia, since the effect is abolished by atropine and paralysing doses of nicotine. Although atropine may be regarded as an almost specific parasympaticolytic drug and the used doses of nicotine probably merely acted on the intestinal ganglia, since they hardly did affect histamine induced contractions (which often are reduced by even moderate paralysing doses of nicotine) the presumed ganglionic site of action of catechol ought to be confirmed by other paralysing drugs.

Cocaine in the used concentrations is believed to act only on the nervous elements of an isolated intestine, especially on the postganglionic nerves. The fact that cocaine inhibits the effect of catechol as well as the effect of nicotine, which according to our present knowledge stimulates the ganglia of the gut, indicates a neural origin of the catechol induced contraction. The findings that cocaine inhibits the effect of serotonin on the guinea-pig ileum agree with previous experience (ROCHA E SILVA, VALLE and PICARELLI 1953, GADDUM and PICARELLI 1957). Hexamethonium is regarded as a highly specific ganglionic blocking substance; its obliteration of the contractions caused by catechol and nicotine forms a rather convincing support of the idea, that catechol, like nicotine, stimulates the intramural ganglia. The inhibiting effect of hexamethonium on acetylcholine produced contractions in some preparations may be explained by assuming that in these strips stimulation of ganglion cells partly contributed to the contraction due to acetylcholine, in agreement with the findings of *Feldberg* (1951).

It seems unlikely that catechol releases other smooth muscle stimulating substances than acetylcholine in the gut, such as histamine, serotonin or substance P. The effect of histamine is not inhibited by atropine or by moderate concentrations of cocaine or hexamethonium but is rather augmented; moreover the specific histamine antagonist phenbenzamine left the catechol induced contractions unimpaired. The serotonin induced contractions are not affected by hexamethonium, but by BOL, a potent serotonin antagonist (SOLLERO, PAGE and SALMOIRAGHI 1956), which in the present study had no effect on the contractions caused by catechol. The action of substance P is not antagonized by atropine, cocaine, hexamethonium, or nicotine. It might be of interest to compare the effect of catechol on the gut with its effect on the cardiovascular system, where it exerts a cardiostimulant action of sympathetic origin on the intact animal, whilst the effect on the isolated heart is depression (WALTON *et al.* 1959, GATGOUNIS and WALTON 1960).

In view of the findings of BACQ (1936) that catechol sensitizes peripheral organs to the effect of adrenaline, probably because it inhibits the catechol o-methyltransferase (BACQ *et al.* 1959), the antagonism seen in this investigation is rather puzzling. The effect of adrenaline and noradrenaline on the terminal part of the guinea-pig ileum is, however, not purely depressant; thus in preparations maintained in a state of tonic contraction by histamine, MUNRO (1953) found that small doses of adrenaline induced relaxation of the strip, while large doses caused a motor response. It might be possible to explain the present results by assuming that catechol through inhibiting the inactivation of adrenaline and noradrenaline causes a larger but less inhibiting concentration of the catechol amines in the intestinal tissue. In favour of such a view speaks the increase in basal tone of the strip sometimes seen. Another possible explanation is that catechol inhibits the action of adrenaline and noradrenaline through competition with peripheral receptors as found for sympathomimetic amines on the rabbit intestine (ÅSTRÖM 1949).

BOL was kindly put at my disposal by SANDOZ A. G. and phenbenzamine (Lergitin) by the A. B. Recip.

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Influence of Various Factors on Urine Oxygen Tension in the Dog

By

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Abstract

AUKLAND, K. and J. KROG. *Influence of various factors on urine oxygen tension in the dog.* Acta physiol. scand. 1961. *52.* 350—365. — Urine pO_2 (U-pO_2) was recorded polarographically for 2—4 hours in 17 experiments on anesthetized dogs. During air breathing values between 8 and 50 mm Hg were observed. Histamine, papaverine and Regitin invariably increased U-pO_2, with slight and inconsistant changes in total renal blood flow (RBF), renal venous pO_2 (V-pO_2) and urine flow. Single doses of adrenaline and nor-adrenaline i. v. produced a rapid, transient reduction of RBF, V-pO_2 and urine flow, whilst U-pO_2 increased. Rapid i. v. infusion of hypertonic solutions of urea, mannitol or sodium chloride gave a rapid, transient increase of RBF, whilst U-pO_2 was lowered for several minutes. Oxygen breathing produced a marked rise in V-pO_2, whilst U-pO_2 increased slightly or remained unchanged. It is suggested that U-pO_2 is determined by pO_2 in the renal papilla through equilibration across the walls of the collecting ducts. The main determinants are suggested to be 1. the rate of medullary blood flow, 2. medullary oxygen consumption, possibly dependent on the load of sodium to the medulla. Plasma skimming and counter current diffusion of O_2 in the medulla is discussed. The findings are consistent with an independent regulation of cortical and medullary blood flow.

It has been demonstrated by several investigators that the oxygen tension (pO_2) of urine is far lower than the oxygen tension of renal venous blood (SARRE 1937, HONG and RAHN 1957, RENNIE, REEVES and PAPPENHEIMER 1958). The explanation of this finding is not clear. Lowering of urine pO_2 by reducing substances in urine before measurement can be carried out was excluded as

the cause by RENNIE *et al.* (1958). The same investigators showed also that at low urine flow a high degree of equilibration with respect to O_2 may be reached between urine flowing through the ureter and venous blood in the ureteral wall. They found, however, that the error introduced by gaseous exchange in the urinary tract was small when urine flow was higher than 5 ml/min, and demonstrated clearly that the pO_2 of urine leaving the kidney is low (6—60 mm Hg, mean 28 mm).

In anesthetized dogs RENNIE *et al.* (1958) frequently observed spontaneous variations of urine pO_2, sometimes as rhythmical variations. These spontaneous variations could not be correlated to changes in blood pressure or renal venous pO_2, and it was therefore suggested that "urine oxygen tension is dependent upon a labile mechanism governing intrarenal distribution of blood flow". It was therefore of interest to find out how urine pO_2 is influenced by vasoactive substances. Furthermore, possible correlations between urine pO_2, arterial blood pressure, total renal blood flow and renal venous pO_2 was investigated.

Methods

In all 17 experiments were performed in 15 healthy mongrel dogs of either six, weighing from 15—25 kg. Anesthesia was induced by Nembuthal-sodium, 25 mg/kg body weight. Smaller doses of Nembuthal-sodium were added throughout the experiment for maintenance of light anesthesia. The first experiments revealed, however, that doses of 2—3 mg/kg body weight or more, often produced a temporary increase of urine pO_2. In subsequent experiments maintenance doses were therefore reduced to 1—2 mg/kg.

All animals were intubated to insure free air ways. Most experiments were performed with spontaneous respiration of air. In some experiments the effect of oxygen respiration was studied by administration of pure oxygen through an automatic respirator.

The ureter on one side was exposed by a flank incision and cannulated with a polyvinyl catheter. The tip of the catheter was placed at the pelviureteral junction, and the other end connected to the oxygen electrode chamber. A permanent draining catheter was introduced into the bladder to prevent accumulation of urine from the other kidney.

Urine pO_2

The presence of reducing substances in urine and the relatively rapid equilibration with systemic tissue pO_2 in the urinary tract necessitates a rapid registration devise with a minimum of dead space for continuous pO_2 recording. The polarographic method was therefore selected as the most suitable for the problems to be investigated.

The oxygen tension was measured by means of a polarographic electrode similar to that described by KROG and JOHANSEN (1959). The electrode was mounted in a plastic chamber thermostatically controlled by circulating water through an outer jacket. The total volume of the electrode chamber and the catheter was less than 1 ml. To obtain an electrode assembly with rapid response to pO_2 changes, a teflon membrane with a thickness of 0.00025" was used as covering. This, however, resulted in an assembly slightly sensitive to flow. Calibration of the electrode was therefore done before and after each experiment with saline equilibrated with gas mixtures with known O_2 concentration, with a flow rate corresponding to the average urine flow during the experiment.

Within the flow range of 2—15 ml/min employed the error introduced by flow sensitivity was less than 5 %. The "rest current" of the instrument during passage of nitrogen-equilibrated saline was only 2—4 % of the current registrated when areated saline (21 % O_2) was flowing past the electrode. The stability of the system was satisfactory. Continuous reading of urine pO_2 was obtained for 2—4 hours in each of 17 experiments, with less than 10 % drifting in the course of any experiment.

Renal venous pO_2

In 3 experiments the oxygen tension of venous blood was recorded continuously by a similar electrode mounted on a heart catheter (KROG and JOHANSEN 1959) introduced into the renal vein through a jugular vein. As the position of the electrode within the lumen could not be controlled, artifacts produced by blunting against the venous wall could not entirely be avoided. For the same reason the calibration was probably less exact than for the urine electrode.

Renal blood flow

In 5 experiments renal blood flow was measured continuously by an electromagnetic square wave flowmeter[1]. The probe was applied directly on the isolated renal artery. In another 6 experiments effective renal plasma flow was estimated by clearance of para-amino hippuric acid (PAH) in periods of 5—10 min. Arterial blood for PAH determination was drawn in the middle of each clearance period. Urine and plasma PAH was determined by the method of SMITH *et al.* (1945).

Arterial pressure

A brachial artery was cannulated with a polyethylene catheter which was connected to a Statham strain gauge transducer. Continuous pressure recording was obtained by means of a Sanborn recording system.

Infusions

All experiments were performed during high urine flow in order to minimize oxygen equilibration between the urine and the renal pelvis.

In two experiments diuresis was induced by rapid i. v. infusion of 0.45 or 0.9 % saline. In all other experiments osmotic diuresis was produced by i. v. infusion of mannitol (5 to 15 %) or urea (5 or 10 %) in 0.45 % saline, at a constant rate of 8 ml/min. In 6 experiments PAH was added to the infusion in amounts to provide a constant plasma concentration in the region between 0.5 and 2 mg/100 ml. In addition 0.45 % saline was given by drip at a rate adjusted according to urine flow, to compensate for excessive salt and water loss. Urine flow was measured frequently at the outlet of the electrode chamber by means of graded vials.

Administration of drugs

In two experiments week solutions of drugs were infused directly into the renal artery through a thin polyethylene catheter introduced *via* the left carotid artery. In all other experiments drugs were infused into a brachial vein cannulated with a polyethylene catheter. Continuous infusions were provided by an electric infusion pump calibrated to deliver 0.25, 0.8 or 1.5 ml/min.

[1] Manufactured by Kiger-Dennard Associates, Winston-Salem, N. C., U. S. A.

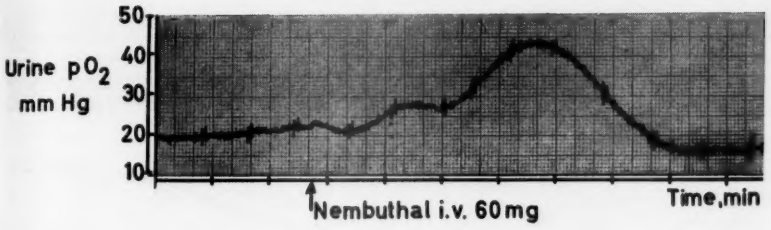

Fig. 1. Effect of Nembuthal i. v. on urine pO_2. Dog ♂, 23 kg. Mannitol diuresis, 12 ml/min.

Results

Control values and spontaneous variations in urine pO_2

During respiration of air urine pO_2 varied between 8 and 50 mm Hg. This large scattering was not only due to individual variations, as it was repeatedly observed that a high urine pO_2 at the start of the recording might change to low levels in the course of the experiment. Alteration in the opposite direction was also seen several times. Spontaneous increase of urine pO_2 by 5—15 mm Hg over a period of 2—5 min appeared in several experiments, at times more or less regularly with an interval of 5—10 min. These variations could not be correlated to changes in arterial pressure, total renal blood flow, renal venous pO_2 or respiration.

Changes in urine flow within the limits studied (2—15 ml/min) seemed not to influence urine pO_2 in any consistent manner. Neither was any consistent difference found between mannitol diuresis, hypertonic salt diuresis or water diuresis. However, our experience with the latter two types of diuresis is limited to two experiments.

High spontaneous urine pO_2 was often associated with low arterial pressure (mean pressure below 100 mm Hg), while low urine pO_2 was most often recorded during high blood pressure. However, exceptions to this rule were observed rather frequently.

Effect of anesthesia

When anesthesia became insufficient as indicated by shivering, movements of the limbs and irregular increase of arterial pressure, urine pO_2 tended to decrease. Maintenance doses of Nembuthal-sodium, 3—5 mg/kg body weight then generally produced a transitory increase of urine pO_2. The response was generally less than that shown in Fig. 1. However, in some experiments a sustained increased level of urine pO_2 was observed after a single dose of Nembuthal.

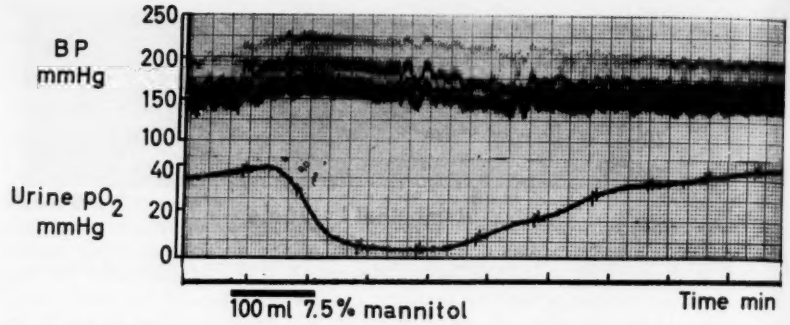

Fig. 2. Effect of rapid i. v. infusion of 7.5 % mannitol in 0.9 % sodium chloride on arterial blood pressure and urine pO_2. Dog ♀, 14 kg. Mannitol diuresis produced by continuous i. v. infusion of 7.5 % mannitol at a rate of 8 ml/min. Urine flow: Control: 8 ml/min. 2 min after rapid infusion: 9 ml/min. 8 min after infusion: 7.5 ml/min.

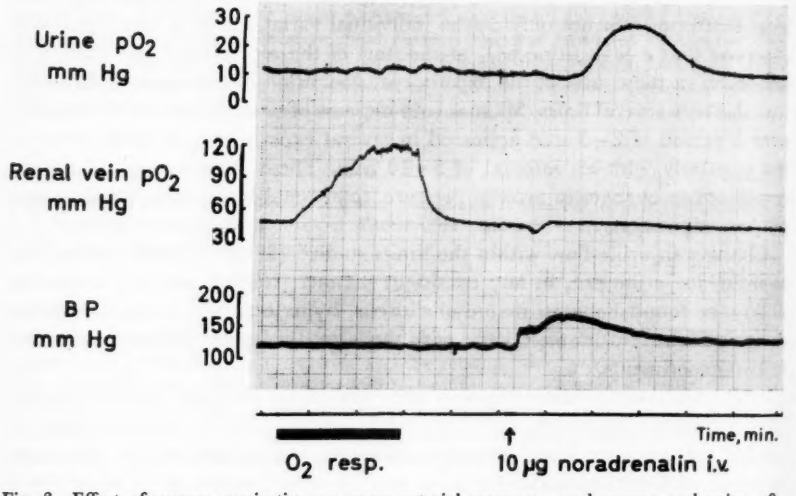

Fig. 3. Effect of oxygen respiration on mean arterial pressure, renal venous and urine pO_2 compared to the effect of i. v. infusion of nor-adrenaline. Dog ♂, 26 kg. Mannitol diuresis, 10 ml/min.

Hypertonic infusions

Rapid intravenous infusion of 20—100 ml 7.5—30 % mannitol, 10 % urea or 5 % saline produced a temporary change of urine pO_2. Most often a slight increase of urine pO_2 of less than 1 min duration was followed by a marked decrease lasting for 5—10 min, while renal blood flow increased considerably for a period of less than one minute (Fig. 2).

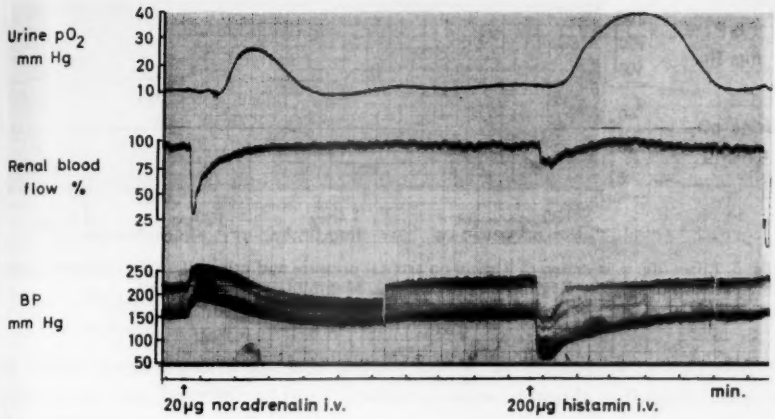

Fig. 4. Effect of i. v. injection of nor-adrenaline and histamine on arterial pressure, renal blood flow and urine pO_2. Dog ♀, 20 kg. Mannitol-urea diuresis, 9 ml/min. Sharp deflection on flow curve at right indicates clamping of renal artery for calibration. (First half of BP curve damped by clot in arterial catheter.)

Effect of breathing pure oxygen

When urine pO_2 was lower than 20 mm Hg, changing between air and oxygen respiration produced no significant change in urine pO_2, whilst renal venous pO_2 increased to high levels during oxygen breathing (Fig. 3). At higher levels of urine pO_2, oxygen breathing produced a moderate increase of urine pO_2 starting after approximately 1 min and reaching maximal value in 4—6 min. In several experiments, however, spontaneous variations in urine pO_2 were larger than those produced by changing between air and oxygen respiration.

Effect of adrenaline and nor-adrenaline

A single i. v. injection of 10—50 μg adrenaline or nor-adrenaline produced invariably (10 injections in 7 different dogs) a transient increase of urine pO_2, starting 1—2 min after the injection. Maximum was observed after 2—6 min, and pre-injection level was reached again after 3—10 min (Fig. 3, 4, 7). In several instances a slight decrease in urine pO_2 of less than 1 min duration preceded the increase. Renal blood flow fell by 25—75 % following the injection and then returned to normal or slightly supernormal values after 1—2 min. The oxygen tension of renal venous blood showed either no change at all or a small decrease of less than 1 min duration. Urine flow decreased markedly immediately following the injection and then returned to pre-injection values or somewhat higher within 1—2 min. The increase in urine pO_2 might thus subsist for several minutes after normalization of total renal blood flow, renal vein pO_2 and urine flow.

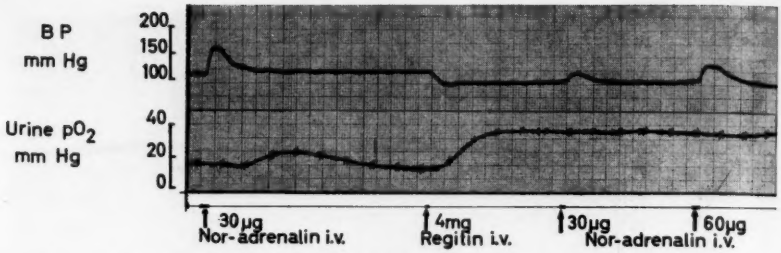

Fig. 5. Effect of i. v. injection of Regitin on arterial pressure and urine $p0_2$. Effect of nor-adrenaline partially blocked by Regitin. Dog ♂, 12.5 kg. Mannitol diuresis, 6 ml/min.

Injection of adrenaline or nor-adrenaline directly into the renal artery in so small doses that almost no systemic effects could be observed, gave principally the same response of urine pO_2 as described above for i. v. administration. It was thus shown that the effect of these substances was direct on the kidney and not mediated via reflexes or produced by alterations in arterial pressure.

Continuous i. v. infusion of adrenaline or nor-adrenaline gave varying results. When urine pO_2 was low, doses of 5—30 µg/min produced an irregular increase of urine pO_2. When infused at higher level, however, both substances tended to cause a decrease of urine pO_2. At intermediate levels the response was quite unpredictable and could not be related to infusion rate.

Continuous infusion of dilute solutions of adrenaline or nor-adrenaline into the renal artery tended to depress urine pO_2. However, also by this mode of administration the response was variable. The intraarterial infusion also reduced urine flow, renal blood flow and renal venous pO_2.

No principal difference between the renal effect of adrenaline and nor-adrenaline were observed.

Effect of Regitin[1]

In 3 experiments Regitin was injected i. v. in doses producing only slight reduction of arterial blood pressure (10—25 mm Hg). A marked, sustained increase of urine pO_2 was observed in all experiments following the administration of 3—4 mg Regitin while renal blood flow did not change appreciably (Fig. 5). Renal venous pO_2 was not measured in these experiments. Regitin reduced the effect of adrenaline and nor-adrenaline on urine pO_2, as shown in fig. 5.

Effect of papaverine

A single i. v. injection of 4—20 mg papaverine produced regularly (6 injections in 3 different dogs) a transient increase of urine pO_2, lasting for 3 to 7 min. This was the case even when the doses employed were too small to

[1] Regitin "Ciba": 2-(N-p-Tolyl-N-oxyphenylaminomethyl)-imidazolini methansulfonas.

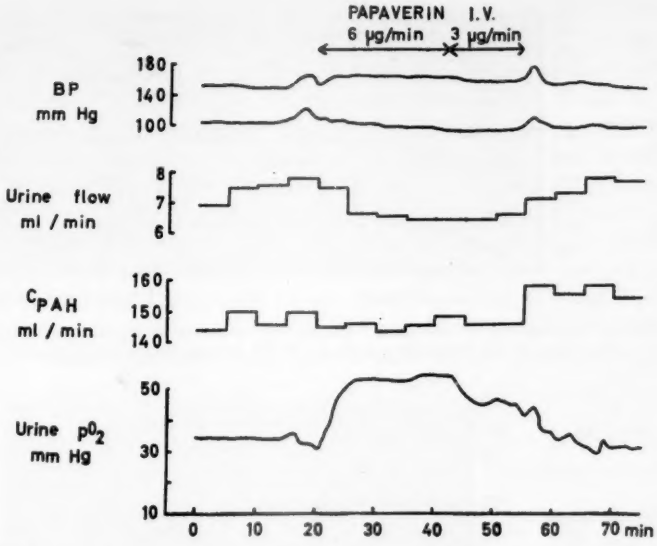

Fig. 6. Effect of continuous i. v. infusion of papaverine on arterial pressure, urine flow, PAH-clearance and urine pO_2. Dog ♀, 22 kg. Mannitol diuresis.

produce appreciable alteration in mean arterial pressure, total renal blood flow or renal venous pO_2.

Continuous i. v. infusion of papaverine at a rate of 2—12 mg/min during 3 periods of 25 to 35 min duration in two dogs, raised urine pO_2 to high levels (40—55 mm Hg). The response was clearly related to the infusion rate (Fig. 6).

Effect of histamine

Rapid, single injections of histamine i. v. produced regularly (8 injections in 6 dogs) a marked increase of urine pO_2, starting within $^1/_2$—1 min after the injection. Maximal values were reached after 2—5 min and pre-injection level was reached after 5—10 min. When doses of 200 µg histamine were used, mean arterial pressure and renal blood flow fell to varying extent for 1—2 min, while renal venous pO_2 decreased by 10—20 mm Hg for an even shorter period of time (Fig. 4,7). With smaller doses, however, these parameters remained practically unaltered, while urine pO_2 increased significantly.

By continuous i. v. infusion of histamine it was possible to maintain urine pO_2 at high levels for seemingly indefinite periods of time. This was consistently found in all 10 infusion periods of 13—65 min duration in 6 different dogs. It was shown by varying the infusion rate between 8 and 60 µg/min that the increase of urine pO_2 was clearly related to the dosage of histamine. A slight

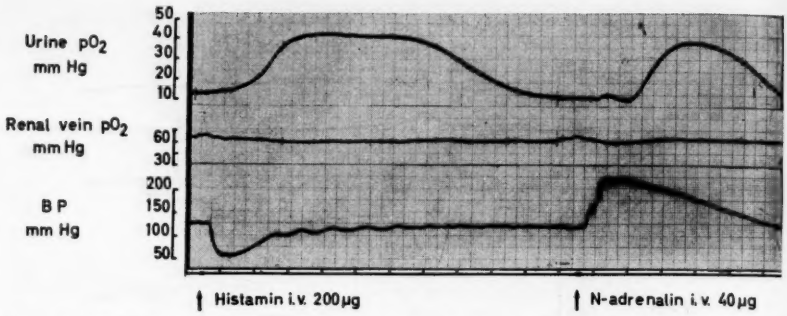

Fig. 7. Effect of i. v. injection of histamine on urine pO_2, renal venous pO_2 and arterial pressure compared to the effect of nor-adrenaline. Dog ♂, 26 kg. Mannitol diuresis 13 ml/min.

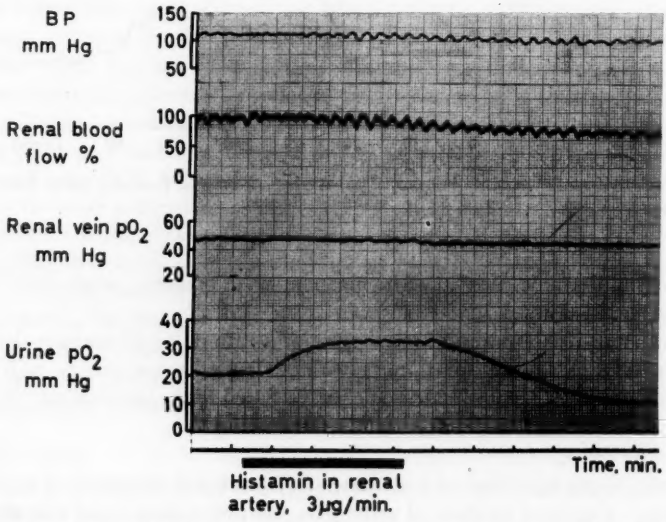

Fig. 8. Effect of histamine infusion into renal artery on arterial pressure, renal blood flow, renal venous and urine pO_2. Dog ♂, 18 kg. Mannitol diuresis. Urine flow gradually declining from 5.0 to 3.9 ml/min.

fall of arterial blood pressure and sometimes also a decrease of renal blood flow was observed during the infusion. By infusion of histamine directly into the renal artery at a rate of 1.5—10 µg/min it was shown, however, that the increase of urine pO_2 could be obtained independently of changes in arterial pressure, renal blood flow and renal venous pO_2 (Fig. 8).

Among the drugs tried in this study histamine was found to be the most reliable agent to initiate and maintain an increase in urine pO_2.

Discussion

The oxygen consumption of the kidney is low compared to the large amount of oxygen made available by the high rate of blood flow to this organ. The oxygen tension of renal venous blood is therefore high, generally in the range of 60—70 mm Hg. The abundance of capillaries in the renal cortex should prohibit any great oxygen tension gradient from capillaries to renal tissue, and it would therefore be expected that the oxygen tension in cortical interstitial water should be nearly as high as in renal venous blood. Furthermore, it has been calculated from Krogh-Hill's diffusion equation that the pO_2 gradient across the tubular wall should be less than 2 mm Hg (RENNIE *et al.* 1958). If the urine pO_2 is determined in the convoluted tubules situated in the renal cortex it would therefore be assumed to have values approximately as high as renal venous blood. In agreement with earlier results, the present experiments have shown that this is not the case. It was found that urine pO_2 is always lower than renal venous pO_2, and furthermore that urine pO_2 can be varied quite independently of renal venous pO_2. These seemingly contradictory statements therefore demand an explanation.

It might be suggested that a large proportion of the renal blood flow is shunted through arterio-venous anastomoses, thereby providing only small amounts of oxygen to the renal tubules, simultaneously producing a high oxygen content of renal venous blood. While this hypothesis might explain some of the findings regarding urine oxygen tension, it is incompatible with the high extraction ratio of substances like diodrast or PAH, as stressed by SMITH (1951). It should also be pointed out that a.-v. shunts have never been anatomically demonstrated in the kidney in spite of careful search (TRUETA *et al.* 1947, VON KÜGELGEN *et al.* 1959).

Based on extensive studies in anesthetized dogs, RENNIE *et al.* (1958) suggested that the low urine pO_2 is due to plasma skimming, providing low hematocrit blood and thereby blood with low oxygen capacity to the peritubular circulation (PAPPENHEIMER and KINTER 1956). Assuming that the degree of skimming could be estimated by the PAH extraction ratio, RENNIE *et al.* (1958) found that urine pO_2 varied inversely with the degree of plasma skimming. The same authors also presented experimental evidence to show that the low urine pO_2 can not be explained by a failure of equilibration across the tubular wall. It should be pointed out, however, that a failure of equilibration would probably not cause a low urine pO_2, because it must be assumed that the pO_2 of the glomerular filtrate is nearly as high as in arterial blood (about 100 mm Hg during air breathing). Oxygen must therefore diffuse out from the tubular lumen to an area with even lower pO_2 than that of pelvic urine.

As an alternative explanation RENNIE *et al.* (1958) mentioned that the urine pO_2 might be determined in the collecting ducts. By rather elaborate calculations based on several assumptions it was found, however, that the urine also

in this case must be equilibrated with tissue supplied with low hematocrit blood. They suggested therefore that a skimming process might provide low hematocrit blood to the medullary circulation. The anatomical circumstances would probably favour an equilibration in the collecting ducts as well as in the convoluted tubules, and several authors have *a priori* assumed that urine pO_2 is determined in this place (SARRE 1938, ULLRICH 1959, LEVY 1959, KRAMER *et al.* 1960). If this hypothesis is correct, it follows that the papillary interstitial pO_2 must be very low, even lower than that of pelvic urine, because the direction of oxygen diffusion must be out from the lumen (*v. s.*). The interstitial oxygen tension in the various zones of the kidney is not known. In a previous study it was shown, however, that the mean oxygen tension is lower in the medulla than in the cortex, compatible with a low pO_2 in the tissues surrounding the collecting ducts (AUKLAND and KROG 1960).

The following factors might be suggested as responsible for a low papillary oxygen tension:

1. Low papillary blood flow.
2. Counter current diffusion of oxygen across the loops of the vasa recta.
3. Alkaline pH within the papilla with resulting high hemoglobin oxygen binding capacity at low pO_2.
4. Plasma skimming providing low hematocrit blood to the medullary circulation.
5. High medullary oxygen consumption.

Some aspects of these factors will be discussed in the following.

1. Small amounts of oxygen may be brought to the papilla by urine both *via* the loops of Henle and *via* the collecting ducts. The flow rate of urine in the loops of Henle and the flow rate of blood in the vasa recta have been shown to be of the same order of magnitude (LILIENFIELD, BAUER and MAGANZINI 1959, KRAMER *et al.* 1960). However, due to the low oxygen capacity of urine compared to that of blood it must be assumed that the main oxygen supply is *via* the vasa recta, thus depending on the flow rate in these vessels. The extraordinary length of the medullary capillaries (vasa recta) would *per se* indicate a low oxygen supply to the medulla, and various estimations have also shown that the blood flow itself is low as compared to cortical blood flow. A slow accumulation of thorotrast in the rabbit medulla and of labelled albumin in the papilla of dogs has been demonstrated (TRUETA *et al.* 1947, LILIENFIELD *et al.* 1959). SCHIEVE *et al.* (1959) found by thermometric methods that the blood flow in the inner portions of cortex was 25 times greater than in the middle of the inner medulla. Similar results were obtained by measurement of dye appearance time by KRAMER *et al.* (1960), who stated that the blood flow of the papilla per g tissue was about one twentieth of that in the renal cortex. The concept of a relatively low blood supply to the renal medulla as compared to that of the cortex seems thus well established.

2. It has also been suggested that the oxygen supply to the papilla is further limited by the counter current flow in the vasa recta (LEVY 1959). It must be assumed that oxygen will diffuse from the descending to the ascending portion of the vessel loops according to pO_2 gradients. The quantitative significance of this shortcircuiting of oxygen will therefore be greatest when arterial pO_2 is high as during oxygen breathing, and even greater during oxygen breathing at 2.5 atm. pressure, as used in the experiments of RENNIE *et al.* (1958). As mentioned by ULLRICH (1959) it is possible that this mechanism invalidates the calculation of the dynamic hematocrit of the region where urine pO_2 is determined (RENNIE *et al.* 1958).

The extent to which the counter current exchange diffusion will reduce the supply of oxygen to the medulla will depend upon the rate of oxygen diffusion across the vessel loops and on the flow rate in the vessels. In short time experiments no changes in the diffusion coefficient would be expected. The oxygen supply to the medulla should therefore depend upon the blood flow in such a manner that an increase of blood flow would result in more than proportional increase of oxygen supply.

3. The pH of papillary interstitial fluid is not known. The low effective blood flow to the medulla with respect to highly diffusible substances would favour the accumulation of acid metabolites. On the other hand, secretion of hydrogen ions into the collecting ducts within the papilla (ULLRICH, EIGLER and PEHLING 1958) should tend to increase pH of interstitial fluid surrounding the collecting ducts. The pH might thus be expected to be determined by the relative magnitude of these two opposing factors. In dogs breathing oxygen, KRAMER *et al.* (1960) found no increase of papillary hemoglobin oxygen saturation after injection of cyanide into the renal artery. They concluded therefore that the blood in the medullary circulation is nearly completely saturated with oxygen during oxygen breathing. It may be questioned, however, if a single injection of cyanide will reach the inner medulla in sufficient amounts to stop respiration effectively, as it must be assumed that the penetration of cyanide into the inner medulla will be greatly impeded by the counter current exchange mechanism. As far as we can see, this finding is only compatible with the very low urine pO_2 if blood within the papilla is strongly alkaline compared to systemic blood. On the other hand, the slight or lacking effect on urine pO_2 of oxygen breathing would indicate that the hemoglobin in the papilla is not nearly saturated with oxygen.

Although we can not exclude that an alkaline reaction within the papilla may contribute to provide a low urine pO_2, it seems quite unlikely that the acute variations in urine pO_2 produced by drugs should result from variations in papillary pH.

4. Plasma skimming providing low hematocrit blood in the medullary circulation can not be excluded by the present experiments. It was repeatedly shown, however, that great variations in urine pO_2 could be produced without

significant alterations in arterial pressure or renal blood flow, factors which should determine the degree of skimming. The cell separation theory thus seems insufficient to explain our findings.

5. The oxygen consumption of the inner medullary zone (including the papilla) has been found to be low compared to cortical oxygen consumption, both *in vitro* (For references see ULLRICH 1959) and *in vivo* (KRAMER *et al.* 1960). Evidence has also been presented to show that the papillary oxygen consumption is directly dependent on the hemoglobin oxygen saturation within the papilla (KRAMER *et al.* 1960). The effect of variations in papillary blood flow on urine pO_2 should therefore be buffered by a varying rate of oxygen consumption in the papilla.

The oxygen consumption of the outer medullary zone has been shown to be high compared to that of the inner zone (For references see ULLRICH 1959). As the capillary loops of the inner medulla also traverse the outer medullary zone, the oxygen consumption of the outer zone might determine pO_2 in the inner zone, and thereby also urine pO_2. It has recently been suggested that the main part of renal oxygen consumption is concerned with, and parallels tubular sodium reabsorption (HESS THAYSEN, LASSEN and MUNCK 1960, KRAMER and DEETJEN 1960, KIIL, AUKLAND and REFSUM 1961). The demonstration of a low sodium concentration in the first part of the distal convoluted tubules (GOTTSCHALK and MYLLE 1959), shows that sodium is avidly reabsorbed in the loops of Henle, and then probably by the wide portion of the ascending limb of the loops. ULLRICH and PEHLING (1958) found that the oxygen consumption of slices from the outer medulla increases linearly with sodium concentration in the medulla in the range of 0—450 meq/l, and suggested that this was due to increased sodium transport by the cells of the wide ascending limb, which is localized in the outer medullary zone. It is thus possible that an increased load of sodium to the wide ascending limb, brought about by increased glomerular filtration rate or by osmotic diuresis, might increase sodium transport and oxygen consumption of the outer medullary zone, and thereby reduce papillary and urine pO_2.

From the general discussion above it appears that acute changes in urine pO_2 is most likely due either to changes in papillary blood flow, and/or to changes in outer medullary oxygen consumption. If papillary blood flow is the main determining factor, the present findings show that papillary blood flow may be varied widely independent of the cortical blood flow. (For practical purposes the total renal blood flow may be taken as a measurement of cortical flow, as the cortical circulation probably receives about 98 % of the total blood supply to the kidney (KRAMER *et al.* 1960).)

An independent regulation of cortical and medullary blood flow is by no means a new hypothesis. TRUETA *et al.* (1947) found in rabbits subjected to various traumatic procedures that the cortical circulation might decrease markedly while the flow apparently increased in the medulla. This finding of

diversion of cortical blood flow to the medulla through the juxtamedullary glomeruli has been confirmed by several authors in rabbits, but has not been demonstrated in dogs (For references see SMITH 1951). Recently, however, THURAU *et al.* (1960) found that the medullary blood flow of the perfused dog kidney varied proportional to perfusion pressure, while cortical flow showed autoregulation, *i. e.* constant flow at increasing perfusion pressure. In the present experiments no consistent correlation between blood pressure and urine pO_2 was found, probably due to a direct effect of the drugs on the medullary circulation.

GRUPP (1957) studied the distribution of renal blood flow by temperature measurements in the dog kidney. High doses of adrenaline i. v. (40 μg/kg) stopped the renal circulation for 1—2 min. In the following 5—6 min, however, total renal blood flow nearly reassumed control values, while cortical renal blood flow was still greatly reduced. He concluded that during these conditions a high proportion (at least 50 %) of the normal renal blood flow might pass the kidney in spite of ischemic renal cortex. In the present study a marked increase of urine pO_2 following injection of adrenaline or noradrenaline might directly suggest an increased medullary blood flow. It is difficult to offer any pharmacological explanation to this finding, however. While it is well known that adrenaline may produce vasodilatation, no such effect has been recorded for nor-adrenaline (GOODMAN and GILMAN 1955). As the nor-adrenaline preparation used contained less than 4 % adrenaline, it is unlikely that the increase of urine pO_2 following injection of nor-adrenaline was due to admixture of adrenaline. This was also indicated by nearly identical response to equal doses of adrenaline and nor-adrenaline. As we would hesitate to propose a vasodilatating effect of nor-adrenaline in the medullary circulation, it seems more reasonable to suggest that the rise of urine pO_2 is due to a temporary reduction of glomerular filtration rate with resulting decrease of oxygen consumption in the outer medullary zone. It might also be suggested that the variable response to adrenaline and nor-adrenaline results from the interaction between two antagonistic effects: 1. Increased vascular resistance in the medullary circulation, reducing blood flow and 2. Reduced oxygen consumption due to reduction of glomerular filtration rate.

The increase of urine pO_2 following injection of adrenaline and nor-adrenaline might also be explained on a hemodynamic basis if these substances had a larger vasoconstrictor effect on the cortical than on the juxtamedullary circulation. Reduction of blood flow to the cortex would increase the side pressure in the interlobular arteries and thus increase perfusion pressure in the afferent arterioles of juxtamedullary glomeruli, originating at right angles from the first portion of the intralobular arteries (TRUETA *et al.* 1947). Medullary blood flow would thus vary inversely to cortical blood flow. A temporary increase in systemic hematocrit due to emptying of splenic erythrocyte reservoirs might

possibly also contribute to the increase in urine pO_2 observed after adrenaline and nor-adrenaline injections.

The consistent increase of urine pO_2 resulting from vasodilatating drugs such as histamine, papaverine and Regitin, without concomitant increase of total renal blood flow, would suggest a high vasoconstrictor tone in the medullary circulation in anesthetized dogs, in contrast to the cortical circulation. The same explanation might also apply to the increase of urine pO_2 produced by Nembuthal. Although variations in sodium load to the medulla might contribute to change urine pO_2 also in these experiments, it has been shown that papaverine and histamine may produce a marked increase of urine pO_2 with slight or no decrease of the filtered load of sodium (AUKLAND, in preparation). The changes brough about by these agents are therefore most likely due to changes in the rate of medullary blood flow.

The decrease of urine pO_2 resulting from rapid intravenous infusion of hypertonic urea or mannitol can probably not be explained as resulting from increased vascular resistance with lowering of medullary blood flow. This is unlikely because hypertonic solutions generally increase blood flow, and especially since it has been shown that papillary plasma flow increases during mannitol diuresis (KRAMER *et al.* 1960). On the other hand, mannitol and urea probably increases the amount of sodium which is brougth to the thick ascending limb of the loops of Henle, and might thereby increase O_2 consumption, either by increased sodium reabsorption or by a higher oxygen demand by the sodium reabsorbing mechanism in the presence of mannitol or urea (KIIL *et al.* 1961).

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Oxygen Uptake and Pulse Rate while Running with Undetermined and Determined Stride Lengths at Different Speeds

By

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Abstract

KNUTTGEN, H. G. *Oxygen uptake and pulse rate while running with undetermined and determined stride lengths at different speeds.* Acta physiol. scand. *52.* 366—371. — Two well-trained subjects ran to steady-state on a motor-driven treadmill at speeds between 9 and 16.5 km/h with undetermined stride lengths and at speeds between 9 and 11.66 km/h with a determined length of stride. Determinations of oxygen uptake and pulse rate were made during steady-state at each speed. The results indicate that kinetic energy is the predominant factor in running (a nearly rectilinear relationship was obtained between oxygen uptake and velocity to the second power). The increase in pulse rate was nearly rectilinearly related to that of oxygen uptake.

Certain references are found in the literature that report a rectilinear relationship between oxygen uptake and speed of treadmill running in steady-state (CHRISTENSEN and HÖGBERG 1950, HÖGBERG 1952 a). BØJE (1944) reported, however, a curvilinear relationship between the two factors, with the oxygen uptake increasing at a proportionately faster rate with increasing speed. As a great part of the energy expenditure in running is presumably kinetic ($e = {}^1/_2\ mv^2$) in nature, one might expect that velocity to the second power would affect the oxygen requirement and oxygen uptake during running so as to cause a non-rectilinear relationship.

The results of various experiments on level walking support this contention. Most recently, RALSTON (1958) and COTES and MEADE (1960) have reported that, not only is the relationship between energy expenditure and walking velocity curvilinear in nature but that, if energy expenditure is plotted against the square of velocity, a rectilinear relationship is obtained. BOBBERT (1960) has arrived at the same conclusions but refers to the latter relationship as "nearly rectilinear".

In the present work it was postulated that there were two dominating factors involved in the energy cost of running on the level: (1) the factor of kinetic energy and (2) the subject's adjustment of stride lenght in order to adapt himself to each speed. In an effort to determine the effects of these factors on steady-state oxygen uptake and pulse rate, experiments were conducted in which a subject ran at various speeds (a) determining his stride length for himself and (b) a parallel series where he used a pre-determined and unchanging length of stride at the various speeds.

Methods

Two well-trained runners (V. K., age — 22 yrs., ht. — 170 cm, wt. — 74.5 kg and P. K., age — 23 yrs., ht. — 179 cm, wt. — 73 kg) were employed as the test subjects in the experiments. The testing was conducted on a motor-driven treadmill, the subject running continuously for approximately 10 min at each speed. He was considered to be in steady-state after 6 min, at which time a recording of pulse frequency and collections of expired air were made. Pulse frequency was recorded by means of an electrocardiograph, one electrode being attached to the subjet's chest over the lower portion of the sternum, another to the subject's forehead, and a third lead serving as a ground. Expired air, collected by the Douglas bag method, was analyzed according to SCHOLANDER (1947). Two tests were made on each day, with a short period of rest between the two bouts of exercise. The order of running speeds was mixed with respect to high and low speeds so as to minimize any effects of conditioning or fatigue. The experiments were run between 11:00 a. m. and 1:00 p. m. the subject having had nothing to eat since a light breakfast prior to 8:00 a. m.

Three sets of experiments were conducted, one set with subject P. K. and two with subject V. K. (with a six month interval between the latter two). Since the results of the experiments were nearly identical, only the data from the second set of experiments with subject V. K. are presented.

The experiments involving the two running techniques were run concurrently. The experiments began by testing the subject at a speed of 9 km/h, the lower limit of both series, and his stride length was determined by counting the number of steps per minute and converting accordingly. The subject was tested at eleven different speeds between 9 and 16.5 km/h determining stride length for himself ("undetermined" stride lengths) and at nine speeds between 9 and 11.66 km/h in the "determined" series. The lowest speed was selected as approximating the lower limit of true running while the upper limit was set at 16.5 km/h, the highest value at which a true steady-state could be obtained. The subject ran at all speeds in the "determined" series with the same stride length as was determined at 9 km/h (77 cm) by having him run in time to a metronome appropriately adjusted to give the proper number of beats, and thus, strides, per minute. It

Table I. Oxygen uptake, pulse rate, and stride length at various speeds while running with undetermined and pre-determined stride lengths

Speed (km/h)	$\dot{V}O_2$ (l/m)	Pulse (/m)	Stride length (cm)
(Undetermined Series)			
9.00	2.46	140	77
9.75	2.61	141	83
10.50	2.75	145	90
11.25	2.91	148	95
12.00	3.11	160	102
12.75	3.36	168	111
13.50	3.62	176	115
14.25	3.87	179	125
15.00	3.93	183	129
15.75	4.17	188	140
16.50	4.24	190	145
(Pre-determined Series)			
9.00	2.46	140	77
9.33	2.52	144	77
9.66	2.65	147	77
10.00	2.83	154	77
10.33	3.02	164	77
10.66	3.28	166	77
11.00	3.50	175	77
11.33	3.78	180	77
11.66	3.99	188	77

was not possible for the subject to run at speeds higher than 11.66 km/h with strides of 77 cm because of his inability to move the body parts quickly enough to maintain the extremely rapid tempo.

Results

The oxygen uptakes and pulse rates obtained are presented in Table I. When plotted in direct relationship to running speeds, the results for the "undetermined" series were found to present slowly rising curves up to a speed of 14 km/h after which the curves began rounding off as the subject neared maximal values. However, plotting the values against velocity to second power (v^2) as shown in Fig. 1, produced quite different results. In the "undetermined" series a relatively rectilinear relationship was found between oxygen uptake and running velocity (to the second power) up to approximately 14 km/h. As speed was increased above this point, the relationship became definitely curvilinear in nature with the curves rounding off at the higher values. The relationships between pulse rate and velocity to the second power were "s" shaped, with pulse rate increas-

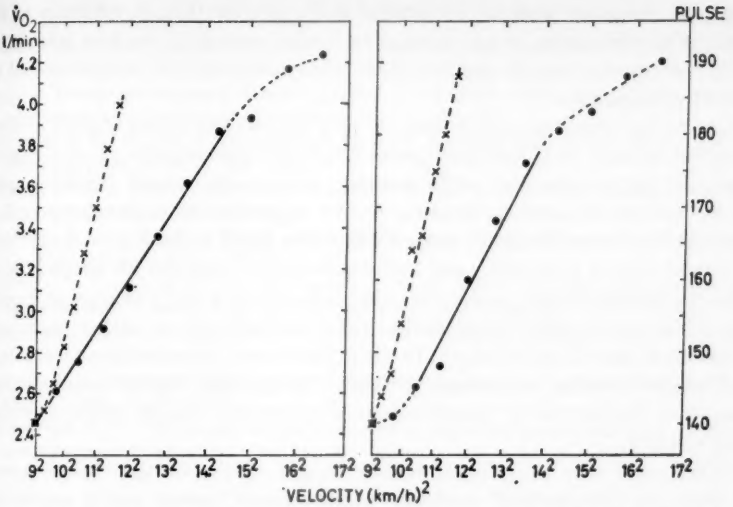

Fig. 1. Oxygen uptake and pulse rate while running with determined (×) and undetermined (•) stride lengths.

ing proportionately faster up to a speed of approximately 14 km/h and after this point, proportionately slower as indicated by the declining slope. In the case of the "determined" series, a linear relationship was found between velocity to the second power and oxygen uptake from 9.66 to 11.66 km/h. The values for oxygen uptake at 9.0 and 9.33 km/h were significantly different from theoretical values obtained from an extension of this linear relationship. The total relationship, therefore, took the form of a "j" shaped curve. A similar relationship was found between velocity to the second power and pulse rate.

Discussion

The fact that plotting oxygen uptake against velocity to the second power resulted in a nearly perfect rectilinear relationship for speeds between 9 and 14 km/h would indicate that running is almost entirely a matter of kinetic energy. This would agree with the results of similar investigations in walking. In the present study, a curvilinear relationship was found at the highest level of running speeds, beginning above 14 km/h. The increase in oxygen uptake (and pulse rate) became smaller per unit increase in running velocity. It is postulated that, because in running the subject is approaching maximal values, the body response to the demands of exercise becomes more and more retarded. It should be mentioned that, while the relationship between oxygen uptake while running in the "undetermined" series and velocity to the second power was "nearly rectilinear"

in nature, it was not perfectly rectilinear. It is suggested that, in addition to the factor of kinetic energy, one or more other factors, such as lift work or additional body movements, enter in and that their relationships should be determined by future investigations.

The energy requirements of running with determined strides slightly shorter than the optimal, as in the lower speeds employed, showed little or no variation from the values obtained while running with undetermined stride lengths. Above 9.66 km/h, however, there occurred a rapid and linear increase in values with the total curve being "j" shaped. HÖGBERG (1952 a) has shown that, when a subject runs at a certain speed and shortens and lengthens his stride lengths from the optium length, minor deviations cause only a slight change in energy cost while increasingly higher energy demands come about with increasingly larger deviations in stride length. In the present study, the deviations from optimal lengths became increasingly greater at increasingly higher running velocities, thus causing the "j" shaped relationship between oxygen uptake and running velocity.

When pulse rate was plotted against oxygen uptake for the various speeds in both the "determined" and the "undetermined" series, nearly rectilinear relationships were obtained. With pulse rate as the ordinate and oxygen uptake as the abscissa, the points plotted for the "determined" series lay slightly higher with respect to pulse rates at various rates of oxygen uptake than did those of the "undetermined" series. One explanation might be a possible state of emotional excitement on the part of the subject at having to run in time to a metronome and at unnatural stride lengths.

With regard to the various curves obtained, the values that seem to be a bit higher than one might expect are those for pulse rate for the lowest running speeds in the "undetermined" series. The reason for this is not understood, but it is suggested that one cause could be the hydrostatic effect, which the increased milking action of the muscles helps to overcome at the higher running speeds.

The increase in stride length of the subject was found to be directly related to the increase in running speed in the "undetermined" series. The rectilinear relationship obtained from this test series coincided with the rectilinear relationship obtained from a single experiment undertaken as a check. The subject increased his velocity from 9 km/h to 16,5 km/h by increasing his stride length and not by increasing his stride frequency (there was actually a slight decrease in frequency). These two results, the relationship of stride length to running speed and the subject's method of increasing his velocity are both in agreement with those of HÖGBERG (1952 a). The results of OGASAWARA (1934) also indicated an increase in stride length as the means by which a runner increases running speed, but a relatively large scattering of values was reported. The consistency of the results of the present study and of HÖGBERG are quite likely due to the greater controls of experimental design and the degree of training of the subjects.

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Cholinesterases in Human Spinal Fluid and Brain[1]

By

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Abstract

SVENSMARK, O. *Cholinesterases in human spinal fluid and brain.* Acta physiol. scand. 1961. *52.* 372—378. — By electrophoresis and chromatography on DEAE-cellulose it was shown that human spinal fluid and brain tissue extracts contained three cholinesterases: Two butyrylcholinesterases and one acetylcholinesterase. One of the butyrylcholinesterases had the same electrophoretic mobility as serum cholinesterases. The other butyrylcholinesterase moved together with the γ-globulin and the acetylcholinesterase together with the τ-globulin fraction of spinal fluid. These findings indicate that the acetylcholinesterase in spinal fluid derives from brain whereas it can not be decided whether the butyrylcholinesterases originate from plasma or from brain. The low mobility fraction of butyrylcholinesterase may arise from the action of sialidase.

Human spinal fluid catalyzes the hydrolytic splitting of benzoylcholine and acetyl-β-methylcholine indicating the presence of butyrylcholinesterase (pseudocholinesterase, unspecific cholinesterase) and of acetylcholinesterase (specific cholinesterase). The rates of splitting are about 1/200 of the rates at which the choline-esters are split by human plasma and erythrocytes (GLASSON and MUTRUX 1946, REISS and HEMPHILL 1948).

In human brain both types of cholinesterases occur (ORD and THOMPSON 1952); the butyrylcholinesterase is probably located in glia and SCHWANN cells (CAVANAGH, THOMPSON and WEBSTER 1954). In rat brain acetylcholin-

[1] Preliminary report: SVENSMARK (1958).

esterase is associated with the cell structure and only small amounts occur in solution. Butyrylcholinesterase is found mainly in the cytoplasma (HOLMSTEDT and TOSCHI 1959). The acetylcholinesterase in spinal fluid might thus derive from brain and the butyrylcholinesterase from either brain or plasma or from both. A comparison of the electrophoretic mobility and chromatographic behavior of cholinesterases might indicate whether there are differences between the enzymes occurring in the spinal fluid, brain and plasma, *i. e.* whether spinal fluid cholinesterases can originate from plasma or brain.

Materials and Methods

Samples of *human spinal fluid* were obtained by lumbar, ventricular and cisternal puncture from neurosurgical, neurological and psychiatric patients. Small samples (5—15 ml) were concentrated at 0° C by vacuum dialysis (MIES 1953) and larger samples or pools by dialysis against 30 per cent polyethylene glycol (Carbowax 20 M, approximate m. w. 20,000, KOHN 1959).

Human brain tissue was obtained 24 hours after death (traffic accidents (3 brains), coronary occlusion (1 brain)). Grey matter, white matter and mixed tissues from one hemisphere were cut into small pieces (0.5 ml), rinsed in icecold 0.9 per cent sodium chloride and blotted on filter paper. The tissues were homogenized in a Waring blendor with icecold 0.9 per cent sodium chloride containing 40 mM/l NaH_2PO_4 adjusted to pH 7.4 with 0.1 N NaOH (1 liter per kg wet weight of the tissue). The homogenates were kept for one hour at 0° C and centrifuged at 8,000 × *g*. The extraction was repeated twice on the precipitate, and the supernatants were combined. The turbidity of the extract could be reduced by freezing to — 20° C and subsequent thawing and centrifugation. This procedure was repeated three times and the extract finally centrifuged half an hour at 30,000 × *g*. The supernatant was concentrated against 30 per cent carbowax 20 M, dialyzed for 24 hours against the buffers used in electrophoresis and chromatography and adjusted to a protein concentration of 50 mg/ml.

Human serum was obtained from donors without signs or symptoms of disease.

Paper electrophoresis was performed with an LKB apparatus (LKB Fabriksaktiebolag, Stockholm) with SCHLEICHER & SCHÜLL no. 2043 bmgl filter paper and sodium barbital buffer (pH 8.6, $\mu = 0.075$); 5 V/cm were applied for 18 hours at room temperature. Protein was stained with amidoblack 10 B and the electrophorogram was recorded on a paper strip scanner with logarithmic output.

Column electrophoresis was carried out on a column (13 × 400 mm) of ethanolyzed cellulose (PORATH 1954, FLODIN and KUPKE 1956) in sodium barbital buffer (pH 8.6, $\mu = 0.075$); the eluate was collected in 1.5 ml fractions and the protein concentration determined (LOWRY *et al.* 1951).

For *chromatography* on diethylaminoethyl-cellulose (DEAE-cellulose, EASTMAN KODAK, SOBER *et al.* 1956) the protein was dialyzed against 5 mM NaH_2PO_4 adjusted to pH 7.0 with 0.1 N NaOH. Two ml were applied to the column (10 × 120 mm) and eluted at 3° C with a gradient of increasing salt concentration and decreasing pH (from 5 mM sodium phosphate buffer pH 7.0 to 0.5 M NaCl and 50 mM NaH_2PO_4). The eluate was collected in 5 ml fractions and the concentration of protein determined from the optical density at 280 mμ (Beckman spectrophotometer, model DU). The conductivity and the pH of the effluent were determined (Conductivity Meter CDM 2 and pH Meter PHM 22, Radiometer, Copenhagen).

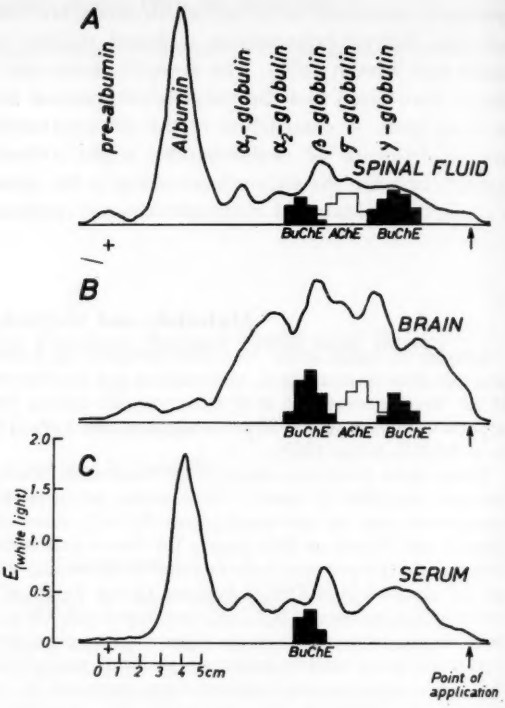

Fig. 1. Paper electrophoresis at pH 8.6 of (A) human spinal fluid, (B) human brain extract (mixed tissue) and (C) human serum. The height of the black columns denotes the activity of butyrylcholinesterase and the height of the open columns that of acetylcholinesterase.

To localize *cholinesterase activity* in paper electrophoresis the wet paper strips were cut into 5 mm transverse sections; these were further cut into small pieces and 0.5 ml of 50 mM NaH_2PO_4 (adjusted to pH 7.4) and 50 μl of 48 mM buturylcholine iodine or acetyl-β-methylcholine chloride were added. The mixture was incubated at 37° C for one hour when butyrylcholine served as substrate and for 8 hours when acetyl-β-methylcholine was used. After the incubation period 450 μl of water were added and the concentration of the choline ester was determined colorimetrically (Hestrin 1949). Corrections for the spontaneous hydrolysis of the substrates were obtained from a sample prepared with water in lieu of enzyme. Eluates from column electrophoresis or from column chromatography were assayed by the same method: 450 μl of the sodium phosphate buffer and 50 μl of the substrate solution were added to 50 μl of the eluate and the assay performed as described above.

To compare the cholinesterase fractions isolated by column electrophoresis their enzymatic activities with different substrates and inhibitors were determined. Benzoylcholine chloride and butyrylthiocholine iodide were used in final concentrations of 4 mM. As inhibitors prostigmin bromide and 10-(α-diethylaminopropionyl) phenothiazin chloride (ASTRA 1397, Astra, Södertälje, Sweden) were used. The inhibitor was dissolved in the 50 mM sodium phosphate buffer and incubated with the sample for 30 min before the substrate was added.

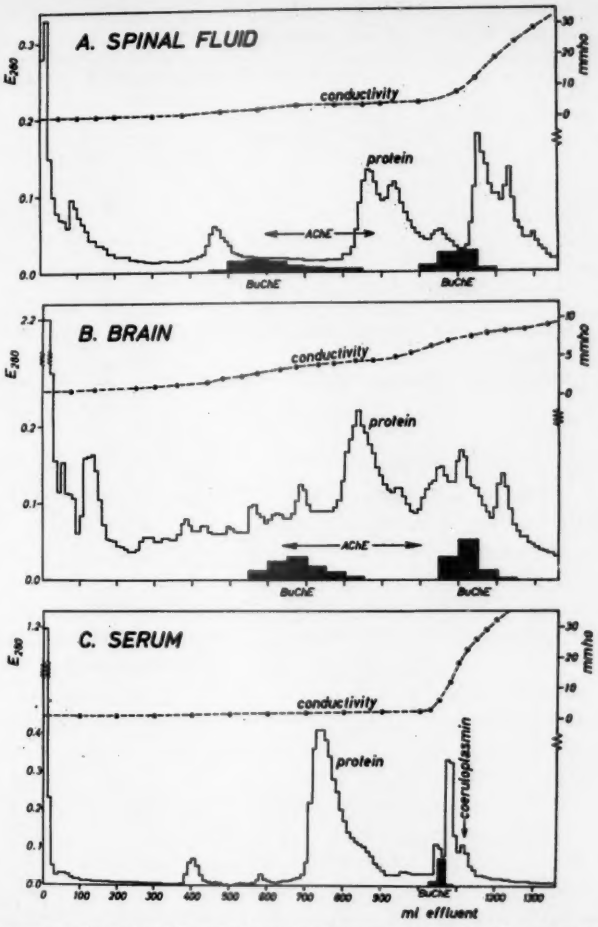

Fig. 2. Chromatography on DEAE-cellulose of (A) human spinal fluid, (B) human brain extract (mixed tissue) and (C) human serum. The height of the black columns indicates the activity of butyrylcholinesterase. The locations of acetylcholinesterase and a greenish protein (coeruloplasmin) are indicated in the figure.

Results

Spinal fluid: Paper electrophoresis at pH 8.6 of concentrated spinal fluid (about 50 mg protein per ml) showed three fractions of cholinesterase: Two butyrylcholinesterases and one acetylcholinesterase. One butyrylcholinesterase was located between α_2- and β-globulin (the α-β-type) and the other together with γ-globulin (the γ-type). The ratio of the activities of the two

Table I. Comparison of the enzymatic activity of the γ-type and the α-β-type butyrylcholinesterases of brain extracts (pH 7.4, 37° C, substrate concentration 4 mM)

Substrate and inhibitor	Cholinesterase activity[1] (μmoles/(hour ml))		Relative cholinesterase activity[2]	
	γ-type	α-β-type	γ-type	α-β-type
Butyrylcholine	3.01	4.39	1.00	1.00
Butyrylthiocholine	2.36	3.19	0.78	0.73
Benzoylcholine	0.38	0.76	0.13	0.17
Acetyl-β-methylcholine	0	0.02	0	0
Butyrylcholine + prostigmin ($4 \cdot 10^{-8}$ M)	1.92	2.96	0.64	0.67
Butyrylcholine + ASTRA 1397 ($6 \cdot 10^{-8}$ M)	2.66	3.90	0.89	0.89

[1] duplicate determinations.
[2] ratio of activity with butyrylcholine as substrate to activity with the substrate in question.

butyrylcholinesterases varied from 1:2 to 2:1 in different samples. The acetylcholinesterase activity was associated with protein located between the butyrylcholinesterases or approximately together with the τ-globulin (Fig. 1 A). This distribution of cholinesterases was seen in 22 different samples of spinal fluid and in 7 pools of spinal fluid.

In chromatography on DEAE-cellulose (Fig. 2 A) one butyrylcholinesterase appeared together with γ- and β-globulins; electrophoresis of this fraction showed that it was identical with the γ-type. The other butyrylcholinesterase appeared after albumin and was identical with the α-β-type. Both butyrylcholinesterases hydrolyzed butyrylcholine but not acetyl-β-methylcholine and were inhibited by prostigmin and ASTRA 1397. The acetylcholinesterase was eluted between the butyrylcholinesterases.

Brain tissue: Paper electrophoresis of the brain extracts showed the same distribution of the cholinesterases as spinal fluid: One butyrylcholinesterase appeared at a site corresponding to that of the α-β-type of spinal fluid and another together with γ-globulin. Acetylcholinesterase appeared between these fractions of butyrylcholinesterase (Fig. 1 B). This distribution of cholinesterases was seen in electrophoresis of extracts from four brains (mixed tissues) and from grey and from white matter of two brains.

The distribution of the cholinesterases in the DEAE-cellulose chromatogram of brain extracts was the same as in spinal fluid (Fig. 2 B).

The two butyrylcholinesterases were isolated by column electrophoresis and the enzyme activity was measured with butyrylcholine, butyrylthiocholine, benzoylcholine and acetyl-β-methylcholine as substrates and with prostigmin and ASTRA 1397 as inhibitors. The activities (calculated in units of the activity measured with butyrylcholine as substrate) were identical in the α-β-type and the γ-type butyrylcholinesterase (Table I).

Serum: The electrophoretic mobility of the serum cholinesterase (GREGOIRE and DERRIEN 1952) corresponded to that of the α-β-type butyrylcholinesterase from spinal fluid and brain (Fig. 1 C). In chromatography on DEAE-cellulose (Fig. 2 C) human serum cholinesterase appeared between albumin and a greenish protein (coeruloplasmin).

Discussion

The butyrylcholinesterase and acetylcholinesterase activities of spinal fluid and brain were associated with different protein fractions. The electrophoretic mobility (pH 8.6) and the chromatographic behavior of the acetylcholinesterase in spinal fluid and in brain were the same. This suggests that part of the spinal fluid protein derives from the brain.

Two butyrylcholinesterases with different electrophoretic mobilities occurred in spinal fluid and brain extracts. The α-β-type had the same electrophoretic and chromatographic properties as plasma cholinesterase and, therefore, it remains undecided whether the occurrence of this enzyme in spinal fluid is due to plasma or brain proteins. The low mobility butyrylcholinesterase, the γ-type, which occurs in both spinal fluid and brain extracts was absent in plasma. This is another indication that spinal fluid contains proteins deriving from brain. It is, however, possible that the γ-type butyrylcholinesterase is produced by the action of sialidase which is present in spinal fluid (SVENSMARK 1961 a, b). The activity of sialidase is so low that its effect on the cholinesterases during the experiments can be neglected. *In vivo* sialidase might reduce the electrophoretic mobility of an α-β-type butyrylcholinesterase secreted from plasma or brain into the spinal fluid. This would give rise to the occurrence of a series of butyrylcholinesterase fractions with mobilities varying between that of the α-β-type and that of the γ-type. That only two butyrylcholinesterase fractions were observed is not necessarily inconsistent with this explanation. On account of the low sialidase activity, the amounts of butyrylcholinesterase with intermediate mobilities might be too small to be detected by the procedures used in this study whereas the non-modified α-β-type and the accumulated sialic acid-free butyrylcholinesterase (the γ-type) might be present in a sufficiently large amount to be determined. Similarly, the occurrence of two cholinesterases with identical enzymatic properties but with different electrophoretic mobilities found in milk and colostrum of swine (AUGUSTINSSON and OLSSON 1959) might be due to the presence of sialidase.

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Acta physiol. scand. 1961. 52. 379—386

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Distribution of Blood in the Arousing Hibernator

By

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Abstract

JOHANSEN, K. *Distribution of blood in the arousing hibernator.* Acta physiol. scand. 1961. *52.* 379—386. — The distribution of circulating blood has been studied in the arousing hibernator following the method of fractional distribution of radioactive indicators (SAPIRSTEIN 1958). The results indicate a conspicuous differential vasoconstriction of the posterior part of the animal during the most active phase of arousal. The blood flow to skeletal muscle in the front of the arousing animals is more than 16 times greater than in the awake non-hibernating animals. The perfusion rate to the myocardium is twice as large in the arousing animal. Likewise the lungs, diaphragm, and brown fat show a substantial increase in blood flow. Blood flow to gastrointestinal tissues is greatly reduced in the arousing animals, whereas flow to the skin, liver and thyroid shows insignificant differences.

During the last 20 years papers on the physiology of hibernation in mammals have been published unabatedly. It has, however, not been possible to study experimentally many facets of this profound phenomenon because the instrumentation and techniques available have disturbed the object of study. This has been particularly true for the study of dynamical cardiovascular physiology in the hibernating and arousing animal. Great progress was made in this field when LYMAN *et al.* (1960) applied the implantation technique for vascular catheters devised by STILL *et al.* (1956), and made successful recordings of blood pressure and pulse pressure in ground squirrels during hibernation and arousal from hibernation. The dramatic and physiologically very active process of arousal from hibernation involves profound changes in cardiovascular parameters like heart rate, blood flow, blood pressure, peripheral

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resistance, etc. Moreover, it has been assumed that extensive changes in the distribution of the circulating blood takes place. This is thought particularly to involve massive constriction of the vascular bed in the caudal part of the animal in the earlier part of the arousal process. Evidence for this assumption was claimed early by MARES (1892) and DUBOIS (1896) using dye injections in an effort to map the distribution of the circulating blood. A number of investigators (LYMAN and CHATFIELD 1950, JOHNSON 1930, BENEDICT and LEE 1938, JOHANSEN and KROG 1959) have tried to demonstrate a differential vasoconstriction by multiple temperature measurements. These studies have confirmed the idea that the front part of the arousing hibernator warms more rapidly than the hind part. In 1950 LYMAN and CHATFIELD demonstrated that the circulating blood was distributed in accordance with this temperature difference. They injected a radio-opaque material directly into the heart and followed its distribution with X-rays. In normal anesthetized animals the injection of Thorotrast outlined, in these authors' view, the whole circulatory system, whereas in the arousing animal only the cranial portion of the circulatory system was visible on the X-rays. All these studies have limitations and are in the author's mind only qualitatively indicative of the distribution of blood. Differences in organ blood flow as well as comparisons of perfusion rates between front and hind portions of the animals are impossible to evaluate with these methods. There seems thus to be no study available which shows quantitatively the distribution of blood in the arousing hibernator. The present study utilizes the method designed by SAPIRSTEIN (1958) for measuring regional blood flow by fractional distribution of radioactive indicators. Comparison is made between one group of awake animals and one group of arousing animals.

Material and Methods

Arctic ground squirrels, Spermophilus undulatus, were used. They were housed individually in wired cages placed at an ambient temperature ranging from 5—10°. Their diet consisted of fresh vegetables, fruit and Friskies cubes. This species, like most other hibernators, hibernates for shorter periods between which it is awake. Two groups of animals were used. One group consisted of animals that had been awake for at least four days consecutively. The other group consisted of animals that had been in hibernation for a minimum of three days.

The method used for measuring the distribution of blood is based on the fact that the uptake of an indicator by an organ is related to the organ blood flow, the arterial concentration of the indicator and the organ's extraction ratio for the indicator. SAPIRSTEIN, studying rats and using K^{42} and Rb^{86} as indicators, found that the organ indicator content became constant 9 sec after intravenous injection and stayed constant up to 64 sec after injection. He found a uniform extraction ratio for all organs during the first 64 sec. It is emphasized that the brain is the only organ showing deviating results from this and is consequently not included in the present study. In the present study only relative values were sought and consequently no integrations done of the arterial concentrations after injection. For details about the method the reader is referred to SAPIRSTEIN (1958).

The awake ground squirrels were anesthetized lightly with Pentobarbital Sodium by the intraperitoneal route. The femoral vein was exposed and cannulated with a polyethylene catheter PE 50. Five μc of $Rb^{86}Cl$ in 0.5 ml saline was then injected and the catheter flushed with heparinized saline. Thirty seconds after injection the animal was killed by injecting 1 ml of a saturated solution of KCl which stopped the heart instantaneously. Postmortem blood was withdrawn for determination of remaining activity. Subsequently the animal was dissected and tissues excised, weighed and counted for activity. For counting, a Nuclear Chicago welltype scintillation counter was used, together with a Nuclear Chicago scaler. Tissues were counted for 5 min at 1,100 volts.

The hibernating animals were moved undisturbed to the operating table. The femoral vein was quickly exposed and cannulated with a polyethylene catheter. The operational procedure lasted from 2—4 min and initiated an arousal in the animal. By the end of the operation the body temperature of the animal had usually risen to 8—10°. The animal was then left in an undisturbed position. The process of arousal continued and when the rectal temperature had reached 15° the isotope was injected and the animal killed as previously described. It was apparent at this time that the process of arousal had entered its most active phase. The oral temperature was more than 15° higher than the rectal temperature. Violent bursts of shivering and muscular activity took place in the front part whereas the hind part was shivering only slightly.

Results

Table I summarizes the results. The activity is expressed as counts per gram tissue per minute or as percent activity recovered of the injected dose. The values are average for 4 or 5 animals in each group.

The most conspicuous difference between the arousing and the awake animals seems to be in the skeletal muscle. In the awake anesthetized animals there was a slightly higher activity in the front than in the hind leg muscles. In the arousing animals, however, the difference was sixfold and more remarkable; the activity of the front leg muscles in the arousing animals was more than 16 times greater than in the awake anesthetized animal. The hind leg muscles of the arousing animals showed somewhat higher activity than the hind leg muscles in the awake animals. This difference was probably related to a slight muscle shivering starting also in the hind part of the arousing animal.

The activity of cardiac muscle from the left ventricle in the two groups was more than twice as great in the arousing animals. This difference was significant to the one percent level and indicates a large increase in coronary perfusion during arousal. The lungs as well as the diaphragm likewise showed a substantially higher activity in the arousing animals. The needs for an increased circulation to these sites is apparent when watching the violent respiratory efforts in an arousing hibernator. The skin taken from the footpads showed a very low activity in both groups, suggesting a general peripheral vasoconstriction during the process of arousal. The activity of tissues from the gastrointestinal system in the two groups demonstrated a markedly reduced

Table I. The table summarizes the results. The activity is expressed as counts per gram tissue per minute or as percent activity recovered of the injected dose. The values are average for 4 or 5 animals in each group. The data has been treated statistically using the method of Siegel

(S. SIEGEL, Non-Parametric Statistics, New York, McGraw-Hill, 1956)

Organ or tissue	Arousing animals		Awake animals		
	Counts/g min.	Percent of inj. dose	Counts/g min.	Percent of inj. dose	P value
Skeletal muscle front part	6,532	2.27	399	0.14	$P < 0.1$
Skeletal muscle hind part	779	0.27	265	0.09	$P < .05$
Skin-front legs	630	0.22	504	0.18	N. S,
Skin-hind legs	446	0.16	446	0.16	N. S.
Heart-ventricular myocardium	16,442	5.73	7,850	2.73	$P < .01$
Diaphragm	8,287	2.89	2,375	0.83	$P < .05$
Kidney	11,700	4.08	33,116	11.55	$P < .05$
Stomach	852	0.30	3,265	1.31	$P < .01$
Small inte tine	243	0.08	3,834	1.34	$P < .01$
Liver	2,211	0.77	2,330	0.81	N. S.
Lung	13,936	4.86	5,835	2.03	$P < .05$
Brown fat	10,850	3.78	1,812	0.63	$P < .01$
Thyroid	5,008	1.75	1,892	0.66	N. S.

activity to these areas in the arousing hibernator. Thus the stomach showed 1/4 the activity of the awake animals. The small intestines in the awake animals similarly showed 15 times more activity than in the arousing animals. The same trend, although much smaller, was also apparent comparing the kidney activities in the two groups. The liver and the thyroid showed small and non-significant differences in their activities comparing the two groups. Of great interest, however, the brown fat excised from the scapular region in the two groups showed more than 5 times as much activity in the arousing animals.

Discussion

The method used in this study is based on the fact that the uptake of indicators by an organ is determined by the integrated arterial concentration of the indicator, the volume flow of blood to the organ, and the organ's extraction ratio for the indicator. We have thus presumably three variable parameters and, when measuring only organ activity, a variance in any of the three parameters would influence the results. In our case, using Rb^{86} as an indicator, it turned out that the indicator content of the various organs stayed constant between 9 to 70 sec after injection of the isotope. The brain was the only organ showing deviating results. Indirectly, this means that every organ has the same extracting ratio within these time limits. If this were not the case,

the recirculating isotope (between 5—10 %) would change the activity in such a way that organs with high extraction ratios would accumulate isotope while organs with low extraction ratios would give it up. In the present study the samples of animals were as much as possible selected on a uniform weight basis. This fact, together with careful dosage of the indicator, justifies the assumption of a reproducible arterial concentration of the indicator. It is emphasized, however, that no extensive measurements were made of the integrated arterial isotope content. Thus the data do not give numerical values of organ blood flow. The data is, however, representative for relative changes in blood flow to organs and tissues.

The results give ample confirmation to earlier findings of a differential distribution of blood between the front and hind part in the arousing hibernator. Earlier evidence for this condition was derived from temperature measurements and more directly from the radiological studies of LYMAN and CHATFIELD (1950). The early studies of Dubois (1896) and MARES (1892) using intravascular injections of various dyes (indigo carmine) indicated that circulation primarily took place in the anterior portion of the animal.

The introduction of a radio-opaque material directly into the heart is technically extremely difficult. In order to get reproducible results the contrast material should be injected in the same heart compartment and under the same pressure in each animal. The difficulty involved in this in an animal the size of a hamster can be easily appreciated. The interesting results presented by Lyman and Chatfield must necessarily be interpreted with these limitations in mind. The X-ray pictures reproduced in their article show a clear delineation of the great thoracic veins 4 seconds after contrast injection in the hibernating animal. This is contradictory to their finding of a systemic circulation time of 10 seconds in the awake animals with many times faster circulation.

One of the more conspicuous characteristics of the hibernators is their ability to arouse themselves from the hibernating condition. The speed and control with which this arousing process takes place have been subjected to repetaed investigation by workers in this field. In the small birchmouse (7—16 g) the whole process of arousal takes place in about half an hour and involves a rise in B. T. of 32° (JOHANSEN and KROG 1959). In other animals studied the arousal takes longer time than this, but is always characterized by a rapid warming of the front part anterior to the diaphragm and a later rewarming of the posterior parts. Many studies have been designed to elucidate the possible sources of heat and the control of the physiological efforts on the part of the animal that makes possible this profound and dramatic phenomenon.

There are two advantages immediately apparent in regard to the selective distribution of blood during arousal. Firstly, it reduces the amount of tissue to be warmed and the surface area for heat dissipation. Secondly, it renders possible a maximum blood flow to organs thought to be involved in the thermogenesis. The outstanding work of DUBOIS (1896) initiated the interest in this

field. His approach was to eviscerate parts of the animals and to study the effects on the awakening process. He also did experiments clamping the aorta or ligating the coeliac trunk and superior and inferior mesenteric arteries. These manipulations did not change the warming pattern of the anterior portion of the body. Tying the vena cava above the liver or tying the portal vein did slow the warming process. Dubois concluded from his experiments that the liver was the predominant source of heat during the warming process. Later studies with evisceration techniques and multiple temperature measurements (LYMAN and CHATFIELD 1950) have questioned the importance of the liver for thermogenesis in the arousing hibernator. The impaired warming after acute portal stasis has later been attributed to pooling of blood in the splanchnic bed rather than interference with the liver as a heat source (BOLLMANN and MANN 1936). The results arrived at in the present study showed no significant change in liver blood flow comparing the awake and the arousing hibernator. This finding supports LYMAN and CHATFIELD's conclusion that the liver has no significant importance as a heat source in the immediate warming process.

The skeletal muscle mass was an early suspect as a source of heat in the arousal process. Thus action potentials have been recorded from muscles in the anterior part at very low temperatures and even before noticeable muscular movements were apparent (LYMAN and CHATFIELD 1950, ANDERSEN, JOHANSEN and KROG 1960). Generally the muscular shivering gets increasingly more violent in the front part until at oral temperatures of more than 30° C the hind leg muscles also come into play. DUBOIS (1896) and later PEMBREY (1901) and JOHNSON (1929) all demonstrated the importance of muscle shivering for the waking process. This has later been substantiated by evidence from work by Lyman and Chatfield. They demonstrated a sizeable reduction in the speed of rewarming after curarization of their experimental animals. The present study demonstrated a remarkable shift in the circulating blood to the skeletal muscle in the front part of the animals resulting in a perfusion rate more than 16 times greater than in the awake animals. It is interesting to note that BENEDICT and LEE (1938) reported that muscle shivering during arousal in the marmot produced more energy than when awake animals were exercised to the limit of their endurance. DUBOIS (1896) stated that the contractions of the diaphragm were of utmost importance in the warming process. JOHNSON (1929) working with the thirteen lined ground squirrel (*Citellus tridecemlineatus*) concluded that the main heat source was the heart and the respiratory muscles. The present data demonstrates a more than threefold increase in perfusion to the diaphragm of the arousing animals. The high activity found in the lungs further substantiates the importance of the increased respiratory efforts in connection with the rapid increase in heat production. Since the lungs represent the only tissue of probable importance for extraction of indicator in the pulmonary circuit, an increase in activity of the

lung parenchyma is suggestive of an increase in cardiac output during the process of arousal.

Special interest is related to the heart as a possible source of heat production during arousal. This muscle is contracting maximally at speeds up to twice the rate ever seen in an awake condition. From the standpoint of rapid transport of heat to adjacent areas the heart and lungs are the only organs in immediate contact with the total cardiac output. On this basis it seems of particular importance that the present study demonstrates a doubling of the activity in the cardiac muscle in the arousing animals. LYMAN and CHATFIELD (1950) apply some interesting logic to the importance of the heart as a heat source during arousal. The conditions prevailing at this time render the heart rather inefficient as a pump leading to particularly high transfer of energy to heat. The particular conditions favoring this are the extremely fast heart rate together with a high peripheral resistance and the high blood viscosity.

The profound changes in blood flow that occur in the arousing hibernator require that blood flow to some sites normally at high perfusion rates must be markedly reduced during arousal. The finding in the present study of a large reduction of the circulation to the gastrointestinal organs seems of special significance in this connection. The 15-times reduction in blood flow to the gut usually receiving 15—20 % of the cardiac output represents a major change in the circulatory pattern.

The tissue most often designated as brown fat has from time to time been suggested to play a vital role in the process of hibernation. The finding of a more than 5 times increase in perfusion rate of this tissue in the arousing animals as compared with the awake animals seems very important. The brown fat in the scapular region was shown in this study to have a blood flow more than 150 times higher than ordinary white fat (omental fat). In addition to the rich vascular supply the brown fat is richly innervated. The observation that the thyroid shows a rather insignificant increase in blood flow compared with the brown fat may be indicative of a crucial role in the arousing process.

There is yet no evidence available pertaining to the control of the conspicuous shifts in blood flow that occur during arousal from hibernation. The present results strongly suggest that the changes in resistance to flow in the various organs are set up locally. The difference in activity of the thyroid versus the brown fat which are largely supplied by the same main arteries serves to illustrate that intrinsic regulation of vascular resistance at the organ or tissue level is the probable effector mechanism for the great shifts in blood flow. The tremendous differences taking place in skeletal muscle should prove interesting from the point of view of arteriolar shunting mechanisms. How these local changes in peripheral resistance are controlled and monitored by the nervous system to accomplish the unique process of arousal from hibernation is completely unknown.

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